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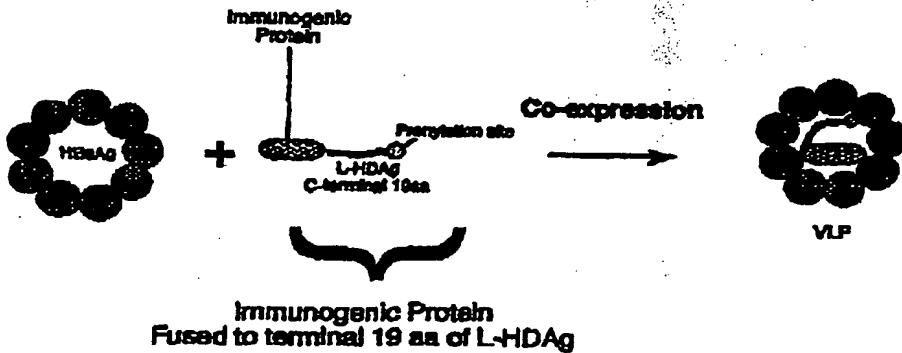
(57) Abstract

The present invention provides a virus–like particle for use in the treatment or prevention of at least a microorganism infection wherein said particle comprises: at least an antigenic and/or immunogenic polypeptide or part thereof from the microorganism, fused to at least the last 19 amino acid of the COOH terminal sequence of the large protein from Hepatitis D virus (L–HDAg), wherein the fusion protein is at least partially enveloped by Hepatitis B surface antigen (HBsAg).

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HEPATITIS DELTA PARTICLE CONTAINING A FUSION PROTEIN IMMUNOGEN

The present invention relates to an improved therapeutic delivery system and in particular to virus-like particles which may be used to ameliorate or prevent infections. For example, the invention relates to virus-like particles which may
5   be used to ameliorate or protect against infections caused by hepatitis B virus and/or at least another hepatitis virus.

Background Art

The immune response to infection with a micro-organism (eg bacteria or viral) is divided into a specific and a non-specific response. The non-specific response
10 becomes effective soon after infection and serves to inhibit spread of the invading organism during the time it takes the host to mount the specific response. In turn, the specific immune response is also divided into 2 components viz. the humoral (antibody) and the cellular immune responses. In general terms, these different responses are effected by different cells of the
15 immune system and although the system involves complex multimolecular interactions, B lymphocytes produce antibodies whereas T lymphocytes are a major component of the cellular response. However, T lymphocytes are also important for the antibody response to infection by providing T cell help.

In the case of antibody production, the specific antigen is recognised by soluble
20 antibody or by immunoglobulin (receptors) on the B cell; the immunogenic activity of the antigen is most often but not exclusively dependent on the conformation of the protein that is recognised in solution. T lymphocyte help is necessary for specific B cell expansion; antigen is taken up by antigen presenting cells (APCs), viz. macrophages and dendritic cells, or by B
25 lymphocytes, presented in context with MHC (major histocompatibility complex) Class II to T helper lymphocytes (CD4+) which then stimulate B cell division. MHC antigens are cell surface glycoproteins which control the recognition of cell and foreign proteins in a complex system of intracellular signalling. The immune

response is dependent on the expression of the MHC, sometimes called the human leukocyte antigen (HLA) system.

If the antigen recognised by the antibody is displayed on the surface of for example a virus particle, then one effect of antibody binding to specific antigen

5   is to neutralise the virus and this in turn results in protection of the host.

In some cases, a viral or bacterial antigen displayed on the surface of a cell can be recognised by antibody and this can result in elimination of the infected cell by a process of antibody dependent cellular cytotoxicity (ADCC). However, the elimination of virus infected cells is most commonly and readily accomplished by

10 CD8+ cytotoxic T cell (CTL). In contrast to the recognition of exogenous soluble antigen, usually processed and presented in a MHC Class II-restricted manner, CTL recognise short (8-11 residues long) antigenic sequential peptides which are MHC Class 1 restricted and which are generally derived from endogenous expression. During cell synthesis, peptides are processed by the cell

15 proteosome machinery then transported to the lumen of the endoplasmic reticulum (ER) by a family of transporter proteins which are encoded in the HLA locus. There, the peptides are examined for the presence of HLA allele-specific binding motifs by MHC Class I molecules. Peptides containing the appropriate motifs are then bound by the MHC Class I protein which then associates with

20 B2-microglobulin and the complex is then transported to the cell surface to be displayed as an integral membrane protein. This complex is then recognised by a CD8+ cell with the appropriate specific T cell receptor (TCR). If, during natural sampling of peptides in the ER, the peptide antigen which interacts with the Class I MHC molecule is derived from a virus or bacterial protein, then this

25 peptide is seen as foreign and the CTL proceeds to eliminate the target cell. In order for this to occur, the target peptide/MHC Class I interaction with the specific TCR is stabilised by several accessory interactions. Elimination of the target cell may be the result of the direct transfer of cytotoxic molecules from the effector cell or by the indirect action of cytokines thought to be TNF-a and IFN-g

30 secreted by the cell.

Although it is possible to recognise MHC Class I binding motifs from the sequence of a protein, it is still not possible to predict precisely if the peptides which encompass these motifs represent antigenic epitopes recognised by a CTL. Furthermore, CTL epitopes, like antibody epitopes, often require T cell

5 help for activity and it is not always possible to recognise T helper epitopes in the amino acid sequence of a protein.

At the present time, 3 types of vaccines are commonly used to prevent infections, namely: i) live attenuated vaccines; ii) killed particle vaccines; and iii) subunit vaccines. The choice can depend on several factors including a

10 knowledge of the specific microorganisms pathogenesis.

Individuals who receive live attenuated vaccines generally require a single injection of the vaccine whereas the use of killed or subunit vaccines requires multiple injections. A major disadvantage of live vaccines is the need for an effective cold chain, otherwise the potency of the vaccine may be diminished,

15 particularly in tropical countries.

In the case of viruses, it is thought that live attenuated virus vaccines are most efficient because humoral and cell-mediated immune responses become activated, although the relative contribution of each has not been determined. In contrast, after vaccination with the latter two preparations, only a humoral

20 immune response usually results because there is no *de novo* synthesis of viral antigens which can enter the endogenous pathway necessary to generate a cellular immune response.

Recent research has shown that vaccination with live attenuated virus will elicit humoral (antibody) and cell mediated immunity (CMI) (ie T cell dependent) and

25 although there are no licenced vaccines at present that are designed solely to elicit CMI, there are a number of examples of successful experimental vaccines which are able to do so. In each case challenge of the vaccinated animal showed complete protection.

The appearance of neutralising antibodies to the envelope proteins of viruses is generally thought to result in the clearance of virus and/or provide a marker of convalescence. In some cases like Hepatitis C virus (HCV) infections neutralising antibodies have not been detected. It is likely that the high mutation rate associated with HCV results in the appearance of antibody-resistant strains of virus that accounts for the co-expression of virus and antibody to the envelope proteins. A practical application of these findings is that passive vaccination with high titre HCV immunoglobulin prepared by cold ethanol fractionation to inactivate residual HCV fails to protect chimpanzees against challenge. These data help to explain why individuals can be re-infected with HCV and lead to the suggestion that a vaccine which is based on a neutralising antibody response is unlikely to be successful against a range of HCV genotypes. Nevertheless, immunisation of chimpanzees with a vaccine based on recombinant E1/E2 protected 5/7 animals from challenge with homologous virus and the disease was ameliorated in the remaining 2 animals (Choo et al., (1994) Proc Natl Acad Sci, USA 91; 1294-1298). However, the chimpanzees were injected on 15 occasions in order for the vaccine to be effective and were only protected against 10 CID_{50}, a relatively small challenge dose, but not 100 CID_{50} (1 CID_{50} is the dose which infects 50% of chimpanzees in a given experiment). Moreover, the duration of protection was very limited. No data are available on the results of challenge of the animals with heterologous virus.

Since the need for and the potential of a vaccine for viruses like HCV that is based on a cellular immune response has been recognised, other workers have chosen to develop a DNA vaccine, based on the finding that direct injection of DNA into animals results in immunisation. Most of these studies reported the development of antibody to the protein encoded by the DNA. Some studies have also reported the development of CTL activity that was able to prevent the growth of a plasmacytoma resulting from injection of a myeloma cell line which constitutively expressed an antigenic protein.

While a lot of research has been carried out into what constitutes an effective prophylactic there remains a need for improved therapeutic agents which are capable of modifying microorganism (including bacterial and viral) infections and which are relatively easy to produce.

5   Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers, but not the exclusion of any other integer or group of integers including method steps.

Disclosure of the Invention

10   The present invention consists of a virus-like particle for use in the treatment or prevention of at least a microorganism infection wherein said particle comprises: at least a antigenic and/or immunogenic polypeptide or part thereof from the microorganism, fused to at least the last 19 amino acid of the COOH terminal sequence of the large protein from Hepatitis D virus (L-HDAg), wherein the

15   fusion protein is at least partially enveloped by Hepatitis B surface antigen (HBsAg).

The antigenic and or immunogenic polypeptide or part thereof used in the invention should be at least capable of eliciting a humoral and/or a T cell response. The T-cell response may be either a T helper cell response or a

20   cytotoxic T-cell (CTL) response. Preferably the polypeptide or part thereof displays a plurality of epitopes. An epitopic region on a polypeptide is generally relatively small - typically 8 to 10 amino acids or less in length. Fragments of as few as 5 amino acids may characterize an epitopic region. Most preferably, some of the epitopes on the polypeptide should be capable of eliciting a humoral

25   response and/or some should be capable of eliciting a T cell response.

In the case of viruses it is generally presumed that a hosts response to viral antigens is almost entirely T-cell dependant. Even the antibody response requires T-cell help. Thus susceptability to virus infection is particularily associated with T-cell dysfunction. Therefore, when the invention is used as a

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prophylactic or therapeutic for viral infections the polypeptide should incorporate at least a range of epitopes which contribute to T-cell activity. Preferably, the polypeptide includes epitopes capable of eliciting a CTL response. Most preferably these epitopes are also substantially conserved between members of
5 the species from which the polypeptide or part thereof was initially selected.

The polypeptide or part thereof used in the invention may correspond to part of a natural protein produced by a microorganism or it may be a recombinant protein which contains at least a antigenic and/or immunogenic peptide. Preferably, the polypeptide or part thereof consists of a plurality of antigenic
10 and/or immunogenic peptides linked together. Most preferably, the polypeptide is selected from regions in a protein or is composed of peptides which have a variety of antigenic and/or immunogenic epitopes and which are substantially homologous between members of the species of microorganism from which the regions or peptides were selected.

15 In addition, the polypeptide or part thereof used in the invention may be selected from any protein from any microorganism (including but not limited to bacteria, protozoa and viruses), provided that the polypeptide or part thereof displays antigenic and or immunogenic properties. By varying the polypeptide or part thereof, different virus-like particles may be produced to treat different
20 microorganism infections without departing from the substance of the invention. Preferably the polypeptide or part thereof is derived from a virus, such as a Hepatitis causing virus.

By way of example only, virus-like particles may be generated against Hepatitis C Virus (HCV). Since peptides with lipid tails (lipopeptides) are well known to
25 stimulate cellular immune responses, it is expected that the lipid component of the HBsAg will have a similar effect, perhaps by enhancing the intracellular delivery of the sub-viral particle. On the other hand, individuals who are already anti-HBs positive may respond most favourably to the sub-viral particle vaccine because the particles will be targeted by the antibody to antigen presenting
30 cells. Furthermore, because L-HBsAg is known to be more immunogenic than

S-HBsAg, incorporation of L-HBsAg into the particles will mimic the second generation of HBV vaccines and lead to improved rates of response to the HBV component of the vaccine.

When HCV is the virus of choice, the polypeptide or part thereof is preferably
5   derived from either the HCV core protein or the NS3 protein. These proteins, in contrast to other HCV proteins, are highly conserved amongst HCV isolates and are known to contain both CTL and T-helper epitopes that are recognised by a range of HLA types. Some examples of CTL epitopes are described in table 1, below.

10   **Table 1**: HCV CTL epitopes in the core protein

HLA Class	CTL epitope location	Reference
A11	aa2-9	Koziel et al., (1993) <u>J Virol</u> 67; 7522 - 7532
B60	aa28-37	Kaneko et al., (1996) <u>J Gen Virol</u> 77; 1305 - 1309
A2.1	aa35-44	Battegay et al., (1995) <u>J Virol</u> 69; 2462 - 2470
B44	aa81-100	Kita et al., (1993) <u>Hepatology</u> 18; 1039 - 1044
A2	aa131-140	Battegay et al., (1995) <u>J Virol</u> 69; 2462 - 2470
A2	aa178-187	Battegay et al., (1995) <u>J Virol</u> 69; 2462 - 2470
DRB1*	aa111-130	Kaneko et al., (1996) <u>J Gen Virol</u> 77; 1305 - 1309
DRB1*	aa161-180	Kaneko et al., (1996) <u>J Gen Virol</u> 77; 1305 - 1309

* CTL epitopes are generally HLA Class I restricted but HLA Class II-restricted CTL have been described (see Kaneko et al, 1996).

In a particularly preferred embodiment of the invention the HCV core protein is selected from any peptide or polypeptide that may be produced from amino
15   acids 1 to 191 of the HCV core protein (wherein the amino acid numbering starts at the first amino acid in the core protein). The peptide or polypeptide must, however, be capable of inducing a T cell response against at least one major subtype of HCV. Preferably the core protein is between about 120 and about 160 amino acids in length. Most preferably the sequence is about 140 amino
20   acids in length.

The length of the polypeptide or part thereof that may be used in the invention is
`   dictated by (i) the length of the amino acid sequence used from L-HDAg and (ii)

the overall length of fusion protein which can be efficiently enveloped by HBsAg. Thus, it will be appreciated that the polypeptide or the length of the amino acid sequence used from L-HDAg may be varied depending on the purpose for which the virus-like particles are being used and the method of construction used.

5   Preferably the amino acid sequence used from L-HDAg is the last 19 amino acids at the COOH terminus of that protein. In such instances the number of amino acids in the polypeptide or part thereof should at least be greater than about 5 amino acids and more particularly about 5 to 500 amino acids in length. Preferably, the polypeptide sequence or part thereof is about 50 to 200 amino

10 acids long. Most preferably the sequence is about 100 to about 160 amino acid long.

If, for example, the polypeptide or part thereof is selected from HCV, the fusion protein which is produced preferably consists of entire core of HCV together with the complete L-HDAg. More preferably the fusion protein contains amino acids

15 1 to 140 from HCV core. In an alternative form of the invention part or all of the HCV core protein may be inserted into an internal site within L-HDAg. For example amino acids 1 to 40 may be inserted into the nuclear localisation site for L-HDAg or into the proline/glycine rich domain.

Preferably the fusion protein is selected from SEQ ID NO 1 to SEQ ID NO 3.
20 The fusion protein selected for use in the invention need not, however, be identical to those described. The fusion protein should, however, be substantially homologous to SEQ ID NO 1 to SEQ ID NO 3, while still maintaining substantially all of the biological activity of the fusion proteins described herein.

25 By "biological activity" is meant at least the ability of the fusion protein to be released inside or from a host cell and the respective polypeptides or parts thereof ability to bind to an appropriate MHC molecule and induce a CTL response against at least one major subtype of HCV. By CTL response is meant a CD8+ T Lymphocyte response specific for an HCV antigen of interest, wherein

30 CD8+, MHC class I-restricted T Lymphocytes are activated.

Various modifications can be effected at non-critical amino acid positions within a polypeptide without substantially disturbing its biological activity. Such modifications include but are not limited to, substitutions (either conservative or non-conservative), deletions and additions.

5   In another embodiment of the invention the polypeptides or parts thereof used in the invention may be modified to enhance substantially their CTL inducing activity. For example it may be desirable to increase the hydrophobicity of the N terminal of a polypeptide or part thereof, particularly where the second residue of the N terminal is hydrophobic and is implicated in binding to the HLA 10   restriction molecule.

With respect to the large form of HDAg, this protein is generated during Hepatitis D virus (HDV) replication. Initially a 195 amino acid protein known as small HDAg (S-HDAg) is expressed that is required for HDV replication and at a later stage, a 214 amino acid protein, the large HDAg (L-HDAg), is expressed 15   that is required for HDV packaging and export. The only molecular difference between S- and L-HDAg is that L-HDAg contains an additional 19 amino acids. The 4 amino acids at the carboxy terminus of L-HDAg represent an isoprenylation site. This site and the preceeding 15 amino acids are however, vital for the interaction between HDAg and HBsAg to permit the packaging event 20   to occur.

While the present invention requires at least the last 19 amino acids of the L-HDAg to be present for the packaging by HBsAg, it will be appreciated that larger forms of the L-HDAg may be present in the fusion protein. Thus any length of amino acids from the L-HDAg may be used in the invention. 25   Preferably, the entire L-HDAg is used in the fusion protein.

The general principle behind the the development of the therapeutic of the present invention is illustrated in Figure 1. Having regard to figure 1 it can be seen that a fusion protein consisting of a polypeptide which exhibits antigenic and or immunogenic properties is fused to at least the last 19 amino acid tail of

the L-HDAg.   The fusion protein is then packaged into virus-like particles through the interaction of the 19 amino acid moiety with HBsAg. This process occurs when the 19 amino acid moiety from the L-HDAg and HBsAg are co-expressed in the same cell.

5   The formation of virus-like particles using the method of the present invention provides a means of stimulating a hosts immune system against HBV and the polypeptide that is fused to the 19 amino acid tail of the L-HDAg. Thus a dual immunological effect is observed from using the method of the invention.

In another embodiment, there is provided in a method for producing virus-like
10   particles containing an antigenic/immunogenic polypeptide or part thereof comprising: incubating host cells transformed with an expression vector containing a sequence encoding a fusion polypeptide containing the antigenic/immunogenic polypeptide or part thereof and at least the last 19 amino acid of the COOH terminal sequence of the large protein from Hepatitis D virus
15   (L-HDAg); in the presence of HBsAg and under conditions which allow expression and packaging of said fusion polypeptide. Preferably the HBsAg is expressed in the same host cells as the fusion polypeptide. This may be acheived by co-tranfection of both expression vectors into the host cells.

The coding sequence for the antigenic/immunogenic polypeptide or part thereof
20   used in the invention may be derived from any source which expresses the polypeptide or part thereof or a protein containing the polypeptide or part thereof. In the case of viruses, for example, the coding sequence for the polypeptide or part thereof or polypetide may be selected from the coding region for coat or envelope antigens, from core antigens or from non-structural
25   proteins. Fragments encoding the desired polypeptides may be derived from cDNA clones or genomic clones using conventional restriction digestion or any other method known in the art. Alternatively the fragments may be obtained by synthetic methods. Once isolated the fragments are then ligated into vectors which contain the coding sequence for at least the last 19 amino acid of the
30   COOH terminal sequence of the large protein from Hepatitis D virus.

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Virus-like particles produced according to the present invention may be expressed in a variety of different expression systems. The selection of the expression system which a researcher wishes to use will to a large extent be based on personal preference. Systems in which the virus-like particles may be

5   expressed include Chinese hamster ovary cells (CHO cells), COS cells, HeLa and MRC-5 cells, all of which have been used in the past to produce vaccines or therapeutic products for use in humans, or any other suitable continuous cell line. The particles may also be synthesised in *Escherichia coli*, in yeast cells or in insect cells infected with recombinant baculovirus. Further, these cells may

10   be used with alternative systems to transient transfection *viz.* stable transfected cell lines, constitutive or inducible expression, expression from a live recombinant virus. In a highly preferred example of the invention the particles are expressed from transient transfection of DNA into COS 7 cells.

The particles may be purified by any protein purification method known in the

15   field. Purification may be achieved by techniques such as, for example, salt fractionation, chromatography on ion exchange resins, affinity chromatography, centrifugation, and the like. See, for example, Methods in Enzymology for a variety of methods for purifying proteins. Preferably they are purified by a combination of sucrose and caesium chloride gradient centrifugation using

20   methods which are well described in the literature.

A number of methods to administer the virus-like particles to uninfected individuals or to infected patients are available. The method of choice to produce the most effective response will however need to be determined empirically and the methods described below are given as examples and do not

25   limit the method of delivery.

Methods for the preparation of therapeutics which contain an immunogenic or antigenic polypeptide or part thereof as the active agent are known to those of ordinary skill in the field. The same preparations can be used with the virus-like particles of the present invention. Typically, therapeutics are prepared as

30   injectables, either as liquid solutions or suspensions; solid forms suitable for

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solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the particles encapsulated in liposomes.

The virus-like particles may be formulated into therapeutics with excipients which are pharmaceutically acceptable and compatible with the active
5   ingredient. Examples of excipients which may be used in such a formulation include, water, saline, ethanol, dextrose glycerol, or the like and combinations thereof. Further, if desired, the virus-like particle formulation may also contain minor amounts of auxiliary substances such as adjuvants, wetting, pH buffering agents, or emulsifying agents which enhance the effectiveness of the vaccine.
10   Suitable adjuvants which may be include in such formulations for example, aluminium hydroxide, N-acetyl-muramy1-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramy1-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(l'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)methylamine (MTP-PE), and RIBI, which contains three
15   components extracted from bacteria, monophosphoryl lipid A trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS).

Virus-like particles may also be formulated into therapeutics as neutral or salt forms. Pharmaceutically acceptable salts include, for example, the acid addition salts (formed with free amino groups of the peptide) and which are formed with
20   inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from in- organic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamins, trimethylamine, 2-ethylamino
25   ethanol, histidine, procaine, and the like.

Virus-like particle formulations may be administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity of virus-like particles to be administered, will generally be in the range of 5 micrograms to 250 micrograms
30   of particles per dose. However this will depend on the subject to be treated, the

capacity of the subject's immune system to respond, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgment of the practitioner and may be peculiar to each subject.

5 Formulations may be administered by the intradermal, subcutaneous or intramuscular routes, or by other routes including oral, aerosol, parenteral, intravenous, intraperitoneal, rectal or vaginal administration. For example the virus-like particles may be administered parenterally, by injection, for example, either subcutaneously or intramuscularly. All the above formulations are
10 commonly used in the pharmaceutical industry and are known to suitably qualified practitioners.

In the case of oral administration, the virus-like particles should be delivered with diluents (water, saline etc) and/or delivery vehicles (tablets, capsules) which do not interfere with the activity of the particles. Oral formulations may
15 include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders

20 Rectal or vaginal administration also requires specific formulation into acceptable forms that contain lubricants and or emulsifying agents. For example such formulations usually include, traditional binders and carriers such as, polyalkylene glycols or triglycerides.

Further, the therapeutic may be given in a single dose schedule, or preferably in
25 a multiple dose schedule. A multiple dose schedule is one in which a primary course of delivery may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at l-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at

least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In addition, the therapeutic containing the virus-like particles may be administered in conjunction with other immunoregulatory agents, for example,
5   immunoglobulins.

Brief Description of Drawings

The present invention will now be described by way of example only with reference to the following non-limiting Figures and Examples.

Figure 1 illustrates the method by which virus-like particles, consistent
10   with the present invention, are made.

Figure 2 illustrates the sequence of the HCV cDNA insert of pA2.

Figure 3 illustrates the sequence of the HCV cDNA insert of pA3.

Figure 4 illustrates the sequence of the HCV cDNA insert of pA10.

Figure 5 illustrates the PCR cloning strategy for clones pA2, pA3 and
15   pA10.

Figure 6 illustrates the DNA and Amino-Acid Sequence of pTBM-HBsAg (ayw3).

Figure 7 illustrates the sequence of the L-HDAg gene.

Figure 8 illustrates the DNA sequence of L-HDAg 19aa tail and alignment
20   with other group 1 isolates of HDV.

Figure 9 illustrates the steps in the construction of plasmid pECE-C/d.

Figure 10 illustrates the general cloning strategy for the construction of the partial core protein expression vectors.

Figure 11 illustrates the sequence of the chimeric insert of plasmid pCδ27core120a.

Figure 12 illustrates the HCV-HDV sequence in plasmid pCδ27core120.

Figure 13 illustrates the HCV-HDV sequence in plasmid pCδ27core140.

5   Figure 14 illustrates the HCV-HDV sequence in plasmid pCδ27core161.

Figure 15 illustrates that a product with a size consistent with the expected size of the core-delta fusion protein was detectable by SDS-PAGE.

Figure 16 provides a western blot of the product of in vitro translated
10   RNA from pCδ27 core120a.

Figure 17 provides a western blot of secreted core/HDAg fusion protein. The figure shows that doublet bands that were identical in size to those detected in cell lysates could be detected in cell culture fluids (CCF) from COS7 cells transfected with pCδ27core140. No HCV core antigen was
15   detected in the CCF from COS7 cells transfected with pCδ27core120 or pCδ27core161. HCV core antigen in the CCF was dependent on co-expression of HBsAg.

Figure 18 is a schematic representation of three HCV L-HDAg fusion constructs according to the invention.

20   Figure 19 is an immunoblot of secreted particles from Cos 7 (lanes 1 and 3) and Huh 7 (lane 2) cells transfected with genes for HCV core 140 full length HDAg fusion protein and HBsAg. Human anti-HDAg was used in lanes 1 and 2 and human anti-HCV was used in lane 3. The arrow indicates the position of the full length chimeric protein.

25   Figure 20 is an immunoblot, with human anti-HDAg, of secreted particles from Huh 7 cells transfected with genes for full length HCV core full

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length HDAg fusion protein and HBsAg. The arrow indicates the position of the full length chimeric protein.

Figure 21 is an immunoblot of secreted particles from Cos 7 cells co-transfected with genes expressing HBsAg and full length L-HDAg containing an internal insertion of a portion of the HCV core protein. The insertions were amino acids 1-40 of the HCV core and were made into the Apa1(nt222) or Sma1(nt490) sites of L-HDAg with (+) or without (-) wild type L-HDAg. Human anti-HDAg was used and the large and small arrows indicate secreted fusion protein and L-HDAg respectively.

Figure 22 provides a graph of BALB/c mice vaccinated with HBsAg-HCV core particle (HBsAg boost, Bs28 treated P815 *in vitro* restimulation).

Further features of the present invention are more fully described in the following Examples. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the invention, and should not be understood in any way as a restriction on the broad description as set out above.

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EXAMPLES

DNA sequencing.

All plasmid DNA sequences reported in this document were sequenced using the Dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer). Unless
5   stated otherwise, all sequences were derived from material cloned into pBluescript KS using the following forward and reverse primers:

primer M13-20-5'-GTAAAACGACGGCCAGT-3'
reverse primer-5'-AACAGCTATGACCATG-3'.

These primers recognise a sequence in pBluescript KS on either side of the
10   multiple cloning site.   The reaction was performed according to the manufacturer's instructions and the extension products ethanol precipitated. The DNA was dried and analysed with the Applied Biosystems 373 sequencer.

Cloning, sequencing and expression of the HCV core gene.

A serum sample from a bone marrow transplant patient with acute hepatitis C
15   diagnosed as anti-HCV positive was used as the source of virus. Briefly, the RNA from the sample was prepared by the addition of guanidine isothiocyanate, sodium acetate and phenol-chloroform, as described (Chomczynski and Sacchi, Anal Biochem 162; 156-159, 1987), incubated on ice for 5-10min and the RNA precipitated by the addition of an equal volume of isopropanol. The sample was
20   then centrifuged and the RNA dried then re-dissolved in distilled water. An aliquot of the RNA sample was mixed with random hexamer primers (Pharmacia), heated to 95°C for 5min then cooled rapidly on ice. The first strand DNA was synthesised by reverse transcriptase (Superscript II, Gibco-BRL) at 37°C for 2h. The second strand synthesis was performed by the
25   addition of DNA polymerase 1 from *Esch coli* (Boehringer, Mannheim), RNaseH (Boehringer, Mannheim) and DNA ligase from *Esch coli* (Boehringer, Mannheim) at 12°C for 1h followed by 22°C for 1h. The preparation was then treated with T4 DNA polymerase (Boehringer, Mannheim) to ensure that the final DNA

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product was completely double stranded. The sample was then phenol extracted, ethanol precipitated, air dried and re-dissolved in distilled water.

The dsDNA sample was then subjected to the sequence-independent single-strand amplification (SISPA) procedure (Reyes and Kim. Mol Cell Probes 5; 473-1991). Briefly, the dsDNA sample was ligated to a dsDNA molecule composed of 2 complementary synthetic oligonucleotides which, prior to re-annealing, were previously phosphorylated by the action of polynucleotide kinase at 37°C for 1h, using T4 DNA ligase (Boehringer, Mannheim) at 15°C overnight then 65°C for 15min. The nucleotide sequence of the complementary primers is:

> RT-A; 3'-TAGCGCCGGCGAGATCTCG-5'
> RT-B; 5'- ATCGCGGCCGCTCTAGAGCTG-3'

The ligation reaction may result in the formation of a dimer of the ds oligonucleotide resulting in reconstitution of an EcoRV site; thus, prior to SISPA amplification with primer RT-A, the product was digested with the restriction enzyme EcoRV. This step ensured that only ds DNA molecules generated by the reverse transcriptase that were ligated to the ds oligonucleotide were amplified.

An aliquot of this SISPA reaction was then amplified by conventional PCR with HCV-specific primers designed to amplify a major region of the core gene ; these primers were designed from the published sequence of the core gene (Okamoto et al., (1990) Jap J Exp Med 69; 167-177). The sequence of the primers is:

> sense primer (#156)   5' - GAGGTCTCGTAGACCGTGCA - 3'
>   (-22 to -3 of 5' UTR)
> anti-sense primer (#155)   5' - CCGGTGCTCCCTGTTGCATAGTTCACG - 3'
>   (residues 1-7 represent sequences designed to facilitate cloning while the
>   remainder represent nt 501 - 482 of the HCV genome)

NB. HCV nucleotide numbering is based on nucleotide number 1 representing the start of the long open reading frame.

The product of this reaction, a 531nt amplicon, was blunt end cloned into pBluescript KS, that was previously linearised with Sma1, and sequenced. This clone was named pA2 and the sequence of the HCV cDNA insert is shown in Figure 2.

Clone A3 was generated in a similar manner, using the SISPA product and primers #157 and #402. The sequence of these primers is:

primer (#157)   5' - CCGGTGCTCGGTCGTCCCCACCACAAC - 3'

(nt 1540 - 1559, excluding 7 nt at the 5' end to facilitate cloning)

primer (#402)   5' - TGGCATGGGATATGATGATG - 3'

(nt 953 - 972).

Primer #157 was designed from an HCV RNA published sequence (Okamoto et al., (1990) Jap J Exp Med 60; 167-177), and primer #402 from a sequence published by Choo et al (Proc Natl Acad Sci, USA 88; 2451-2455, 1991). The product of this reaction, (614 bp) was blunt-end cloned into pBluescript KS linearised by Sma1 to generate clone pA3. The sequence of clone pA3 is shown in Figure 3.

Based on the sequences of clones A2 and A3, primers 1A and 2A respectively were designed to amplify a region corresponding to nt 358-1030. The sequence of these primers is;

primer (#2A) - 5'-TTTCTTGTGGGATCCGGAGT - 3' (1011-1030)

primer (#1A) - 5'-GGTAAGGTCATCGATACCCT - 3' (358-367).

These primers were used to amplify DNA from the products of a SISPA reaction, performed as described above. The product of this reaction was a 673bp fragment that was blunt-end cloned into pBluescript KS that was previously linearised with SmaI, to create clone A10. The sequence of the HCV cDNA insert of pA10 is shown in Figure 4. The HCV sequences in clones pA2 and

- 20 -

pA10 were then ligated to form a continuous cDNA molecule representing nt -22 to 1030 of the HCV genome. This was performed by ligation of the Cla1 fragment from pA2 with linearised pA10. This created clone pA2-A10, the orientation of which was confirmed by restriction enzyme mapping.

5   The PCR and cloning strategy for clones pA2, pA3 and pA10 is summarised in Figure 5.

Cloning of the L- hepatitis B surface antigen gene.

A serum sample from a HBsAg-positive patient represented the source of virus. The virus DNA was purified by protease digestion followed by phenol extraction

10   and ethanol precipitation. Briefly, the virus was pelleted through a 20% sucrose cushion at 39000 rpm for 5h in a Beckman SW41 rotor and the pellet was then mixed with a solution of proteinase K and SDS, and incubated at 37°C for 4h. The solution was then extracted with an equal volume of phenol:chloroform:isopropanol (25:24:1) and the upper aqueous layer containing

15   the virus DNA removed. The DNA was precipitated by the addition of sodium acetate to 0.4M and 2.5vol of absolute ethanol. The DNA was pelleted, dried and re-dissolved in distilled water. The gene for HBsAg was amplified by PCR using primers designed from a published sequence of HBV DNA (Galibert et al., J Virol 41; 51-65, 1982). The sequence of the primers is:

20   Upstream: 5' - ATGGAGAACATCACATCAGGA - 3"
            Downstream: 5' - AATGTATGCCCAAAGACAAAA - 3"

This reaction produced a 681bp product that was cloned into pBluescript KS to create pTBM-LHBsAg and sequenced. The sequence of the HBsAg gene is shown in Figure 6.

25   **Expression of S-HBsAg.**

The region corresponding to the S-HBsAg gene was then excised by digestion of the plasmid with Hind III and sub-cloned into the expression vector pSVL (Pharmacia) to created pSV-HBsAg. Plasmid pSV-HBsAg was transfected into

COS7 and HuH7 cells using the DOTAP procedure. Five days later, the cell monolayers were examined for expression of HBsAg by immunofluorescence and the cell culture fluid for secreted HBsAg by ELISA. The immunofluorescence pattern of the HBsAg expressed in the cells was typically
5   cytoplasmic and the ELISA was positive for HBsAg. These results proved that the HBsAg was not only expressed from pSV-HBsAg but was also secreted.

Expression of L-HBsAg

Coverslip cultures of COS7 and HuH7 cells transfected with pTBM-LHBsAg were examined by immunofluorescence using a monoclonal antibody specific for
10 L-HBsAg. A staining pattern similar to that noted for HBsAg was seen in all transfected cells. Cell lysates were also examined by immunoblot using the same antibody. Two bands of a size consistent with the glycosylated and non-glycosylated forms of L-HBsAg (ie. gp42/p39) were observed in lysates from cells transfected with pSV-LHBsAg but not the control plasmid.

15 **Cloning of the L-HDAg gene**

A serum sample from a HDV RNA-positive patient represented the source of the virus; the RNA was purified by the GIT extraction method described above. The L-HDAg gene was amplified by RT-PCR. The RT step was performed using random hexamer primers (Pharmacia) and the cDNA amplified by PCR using the
20 following primers which include BamH1 restriction enzyme sites to facilitate cloning;

H1- 5' - AAA<u>GGATCC</u>GATGAGCCGGTCCGAGTCG - 3'
H2- 5' - AAA<u>GGATCC</u>TCACTGGGGTCGACAACT - 3'

The product of this reaction was a 465bp amplicon. This was digested with
25 BamH1 and cloned into the BamH1 site of pCDNA3 to create pC-LHDAg1. The sequence of the L-HDAg gene is shown in figure 7.

Expression of L-HDAg.

Coverslip cultures of COS7 and HuH7 cells were harvested 2-3 days after transfection with pC-LHDAg1 and examined by immunofluorescence using a polyclonal rabbit antibody specific for the 19aa terminus of L-HDAg. A

5 characteristic nuclear staining pattern was observed in all transfected cultures. Immunoblot examination of cell lysates from pC-LHDAg1-transfected cells were also positive.

Synthesis of the gene for the C-terminal 19aa of L-HDAg.

The gene for the C-terminal 19aa region of HDAg was assembled from

10 overlapping primers which were designed from examination of the consensus nucleotide sequences of the genomes of group 1 isolates of HDV. The sequence of the primers including restriction enzyme sites to facilitate cloning is:

primer p27 up

-5'-AAAGGATCCTGGGATATACTCTTCCCAGCCGATCCGCCCTTTTCT-3'
15   (BamH1 site)

primer p27 down

-5'-AAACTCGAGTCACTGGGGTCGACATCAGTCGGGAGAAAAGGGCGG-3'
      (Xho site)

The primers were heated to 85°C for 5 min then allowed to re-anneal as the

20 mixture was cooled to room temperature. The product of this reaction was end filled with T4 DNA polymerase (Boehringer, Mannheim) to ensure that the hybrid was completely double stranded. The DNA was double digested with BamH1 and Xho1 and directionally cloned into the corresponding sites in pcDNA3 which was previously digested with these enzymes and this resulted in the

25 construction of pCδ27basic.

The sequence of the gene for the 19aa region (L-HDAg 19a) and an alignment with other Group 1 isolates of HDV is shown in Figure 8.

Construction of HCV core-HDAg fusion proteins.

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i) <u>Full-length HCV core-HDAg</u>

The full length core gene was amplified using primers #156C and #WYH-5 from
pA2-A10; the sequence of the primers including <u>Bam H1</u> and <u>EcoR1</u> restriction
enzyme sites to facilitate cloning is:

5   #156C-5'-CGC<u>GGATCC</u>ATCGAAGGTAGAATGAGCACGAATCCTAAA-3'
         #WYH-5-GGG<u>GAATTC</u>CGGAAGCTGGGATGGTCAAA-3'

The 600bp product of this reaction was directionally cloned into pECE (Leland et
al, (1986), <u>Cell</u> 45; 721-732) which was previously digested with Bgl II and
EcoR1 to create pECE-HCVcore; this plasmid was then digested with EcoR1,
10   made blunt ended by the action of the Klenow component of DNA polymerase 1
(Boehringer, Mannheim) and then digested with Xba1. This was then ligated to
the Sma1/Xba1 fragment from pECEδBE (Chang et al, (1988) <u>J Virol</u> 62; 2403-
2410) that encodes the 51-COOH terminal aa of L-HDAg to create pECE-C/d.
The steps in the construction of this plasmid are shown in Figure 9. The
15   sequence of the junction of the HCV core gene with the HDV antigen gene in
pECE-C/d was determined using primer #1A and the junction of the genes
shown to be in-frame.

ii) <u>Partial core sequences.</u>

The general cloning strategy for the construction of the partial core protein
20   expression vectors is shown in Figure 10.

a) HCV core120a-HDAg

The region corresponding to aa1-120 of the HCV core protein was fused to the
19aa COOH terminus of the L-HDAg to produce a chimeric fusion protein. The
expression plasmid was constructed in the following manner.

25   The region coding for core amino acids 1 - 120 was amplified by PCR from pA2-
A10 using primers TM1 and TM2. The sequence of these primers including the
underlined BamH1 sites to facilitate cloning is:

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primer #TM1

-5'-CGC<u>GGATCC</u>ATCCAAGGTAGAATGAGCACGAATCCTAAA-3'

primer #TM2-

5'-AAA<u>GGATCC</u>ACCCAAATTACGCGACCTACG-3'

5   The product of this PCR was then digested with BamH1 and ligated into the BamH1 site of pCδ27basic to yield pCδ27core120a. The sequence of this chimeric gene was determined and is shown in Figure 11.

    b) HCV core 120-HDAg

The core gene region 1-120 was also amplified from pA2-A10 using primers

10   TM2 and TM3 then ligated to the gene for the 19aa tail of L-HDAg. Primer TM3 is a modified version of primer TM1 that was designed to eliminate a potential loop in the 5' end of the mRNA transcribed from the plasmid, as it was considered that this loop may reduce the efficiency of protein translation from the mRNA. The sequence of primer TM3 including a BamH1 site to facilitate

15   cloning is:

    primer TM3-5'-AAA<u>GGATCC</u>AAAATGAGTACTAACCCTAAACCCCAA-3'.

The product was then digested with BamH1 and ligated into the BamH1 site of pCδ27basic to yield pCδ27core120. The HCV-HDV sequence in this plasmid is shown in Figure 12.

20   c) HCV core140-HDAg

Similarly, the region corresponding to aa1-140 of the HCV core protein was fused to the 19aa COOH terminus of the L-HDAg . The expression plasmid was constructed in the following manner:

The region coding for core amino acids 1-140 was amplified by PCR from pA2-

25   A10 using primers TM3 and TM4 which include a BamH1 site to facilitate cloning. The sequence of the primers is:

    primer #TM3-as above

    primer #TM4-5'-AAA<u>GGATCC</u>GACAAGCGGGATGTACCCCAT-3'.

The product of this reaction was digested with BamH1 and ligated into the BamH1 site of pCδ27basic to yield pCδ27core140. The sequence of the chimeric HCV-HDAg gene in this plasmid is shown in Figure 13.

    d) HCV core161-HDAg

5 The core protein aa1-161 was fused to the 19aa L-HDAg COOH terminus using a similar strategy. The expression plasmid was constructed by amplifying the core gene coding for 1-161 from pA2-A10 using primers TM3 and TM5 which also contain a BamH1 site for ease of cloning. The sequence of these primers is:

10   primer #TM3-as above

      primer #TM5-5'-AA<u>AGGATCC</u>GCCGTCCTCCAGAACCCGGAC-3'

The product of this reaction was digested with BamH1 and ligated into the BamH1 site of pCδ27basic. This yielded plasmid pCδ27core161 and the sequence of the HCV-HDAg chimeric gene contained in this plasmid is shown in
15 Figure 14.

Expression of core-HDAg fusion proteins incorporating 19 amino acid tail of L-HDAg.

<u>a. Methods used for the transfection of DNA</u>

i). Calcium phosphate transfection of HuH7 cells. 6μg of DNA (3ug of each
20 plasmid in the event of co-transfections) was mixed with calcium chloride to a final concentration of 0.12M and hepes buffered saline (HBS) pH7.00, then incubated overnight at room temperature. The solution was then sonicated for 60sec. The transfection mix was added to a 25cm^2 culture flask of HuH7 cells and incubated at 37°C overnight. The cell culture medium was changed the
25 following day and the cells incubated for the desired period.

ii). DOTAP transfection of COS7 and THT1 cells. 10 μg of DNA (5ug of each plasmid) in 100μl of HBS was added to 50μl DOTAP (Boehringer Mannheim) and 50μl HBS, then incubated at room temperature for 10min. The cell culture

medium in a 25cm² flask was replaced with DMEM+1% FCS and the tranfection mixture then added. The cells were incubated at 37°C overnight then re-fed with DMEM+5% FCS and incubated for the desired period.

b. Results.

5   i). Expression from pCECE-C/d and pCδ27core120a . Transfection of pECE-C/d or pCδ27core120a with or without pSV-HBsAg into HuH7, COS7 and THT1 cells resulted in only a few cells which were weakly positive for HCV core antigen. All cells were negative for HDAg. Neither antigen was detected in the cell culture fluid (CCF) after co-transfection with pSV-HBsAg.

10   It seemed likely that two possibilities accounted for the lack of protein expression in these experiments viz. inefficient translation of protein from mRNA or protein stability. To examine these possibilities, in vitro translation in rabbit reticulocyte lysates using in vitro transcribed, capped RNA from pCδ27core120a was performed.

15   A product with a size consistent with the expected size of the core-delta fusion protein was detected by SDS-PAGE in this experiment (Figure 15). This product was subsequently confirmed to be reactive with anti-HCV core by immunoblot. Thus the core-HDAg fusion protein encoded in plasmid pCδ27core120a could be translated normally, suggesting that the poor expression of this plasmid in 20 cell cultures was probably related to protein instability.

ii). Expression from pCδ27core120, pCδ27core140 and pCδ27core161. After co-transfection of these plasmids with pSV-HBsAg into COS7 cells, the intact cells and cell lysates were examined 3 days and 5 days later for HCV core antigen expression by immunofluorescence and immunoblot respectively. The 25 results of the immunofluorescence examination showed that pCδ27core120 expression resulted in weak staining with a diffuse nuclear localisation, while expression from pCδ27core161 was similar except that the antigen was also expressed in the cytoplasm. However, expression from pCδ27core140 showed strong nuclear and cytoplasmic staining. In comparison, HCV expressed from

pECE-HCV core showed strong perinuclear staining.   These results are summarised in Table 2. HDAg was not detected in any of the transfected cells.

These results were confirmed by immunoblot examination of cell lysates harvested on day 5 post transfection. No signal was detected after transfection with pCδ27core120 and only a weak signal detected from pCδ27core161. In contrast, a strong reactive band corresponding to the predicted size of the fusion protein was detected in cell lysates after transfection with pCδ27core140 (Figure 16 and Table 2).

Table 2 Summary of results with pCδ27core120-161

Results with partial HCV core/HDAg fusion proteins			
Construct	HCV core detection in cells by IF	HCV core detection cell lysates	Secreted virus like particles
pCδ27core120	±	-	-
pCδ27core140	++	+++	+
pCδ27core161	+	+	-

Note: no virus like particles detected if the HBsAg gene was ommitted from the transfection mixture

CCF from the transfected cells were then examined.   The samples were clarified, then centrifuged through a 20% sucrose cushion at 38000 rpm for 5h at 4°C in a Beckman SW-41 rotor. The pellets were dissolved in SDS-PAGE loading buffer then examined by immunoblot to detect secreted HCV core antigen.

Weak doublet bands that were identical in size to those detected in cell lysates were detected in cell culture fluids from COS7 cells transfected with pCδ27core140 (Figure 17). No HCV core antigen was detected in the CCF from COS7 cells transfected with pCd27core120 or pCδ27core161. These results

were reproducible. Moreover the data clearly showed that the appearance of the HCV core in the CCF was dependent on co-transfection with pSV-HBsAg. Thus this experiment provides strong evidence that the HCV core protein was secreted from the cells as virus-like particles.

5   The above recombinant DNA techniques are in common use and are described in detail in Sambrook et al (Cold Spring Harbor Laboratory Press, 1989).

Virus-like particles constructed according to the examples using a fusion HCV/HDV protein and HBsAg were immunogenic with respect to HCV and HBV. That is the virus-like particles are capable of stimulating HLA class I restricted

10   CTL responses against the core protein for HCV and the surface antigen of HBV.

Constructs including full length L-HDAg with and without insertions

Schematic representations of the following constructs are illustrated in Figure 18.

15   (a) pCc140FLδ

The gene encoding aa 1-140 of HCV core was excised by PCR from plasmid pCδ27c140 (aa 1-140 fused to C-terminal 19aa of L-HDAg) by BamH1 digestion and ligated into the BamH1 site of a pCDNA derived plasmid containing full length L-HDAg cloned between BamH1 and Xba1 sites. The HDAg insert for the

20   latter plasmid (pCδ27/FL) was amplified by PCR (template pSV27 = complete L-HDAg gene inserted into the Sma1 site of pSVL [Pharmicia]) using primers

(i) HDAg For

5'-AAAGGATCCGGAATGAGCCGGTCCGAGTCGAGG-3'   (BamH1   site underlined)

25   (ii) HDAg Rev

5'-AAATCTAGATCACTGGGGTCGACAACTCTG-3' (Xba1 site underlined)

(b) pCFLcore+δ

The full length gene for HCV core was amplified by PCR from the Australian HCV isolate (in plasmid pECEcore) and blunt-end cloned into the BamH1 site of plasmid pCδ27/FL (see above).

5 The upstream primer for the PCR was the same as for the C140 insert. The downstream primer was as follows:

5'GCG<u>AATTCC</u>GGATCCTGGGATGGTCAAACA-3' (EcoR1 site is underlined).

(c) pSV27-C40Apa1 and pSV27-C40Apa1

These plasmids contain aa 1–40 of HCV core (amplified by PCR) inserted into 10 the Apa1 or the Sma1 sites respectively of full length L-HDAg in plasmid pSV27 (see above)

<u>Primers</u>

(i) Apa1 insertion

HCV-C40Apa1 For

15 5'-AAA<u>GGGCCC</u>GAATGAGCACGAATCCTAAACCT-3'

HCV-C40Apa1 Rev

5'-AAA<u>GGGCCC</u>TGCGCGGCAACAGGTAAAC-3'

Apa1 sites are underlined

(ii) Sma1 insertion

20 HCV-C40EcoR5 For

5'-CTGA<u>GATATC</u>ATGAGCACGAATCCTAAAC-3'

HCV-C40EcoR5 Rev

5'-TTAA<u>GATATC</u>GCCCCTGCGCGGCAACAGG-3'

EcoR5 sites are underlined.

CTL assays

5   Transfected 2X 175cm^2 of Cos 7 cells with pCc140FLδ plus pSVHBsAg using DOTAP. Culture fluids were harvested 5 days post transfection and the VLPs centrifuged through a 20% sucrose cushion.

Transfected 2X 175cm^2 of Cos 7 cells each with pCc140FLδ (plus pSVHBsAg) and pCδ27c140 (plus pSVHBsAg) using DOTAP. Culture fluids and cell lysates

10   were harvested 5 days post transfection. VLPs were prepared as above. Samples from transfection using pCc140FLδ were also analysed by western blot (Figure 19).

Transfected 2X 175cm^2 of Huh7 cells by Calcium Phosphate co-precipitation with pCFLcore+δ plus pSVHBsAg. Culture fluids were harvested 5 days post

15   transfection. VLPs (prep as above) were examined by western blot (Figure 20).

Internal insertions into HDAg

1x25cm^2 flasks each of Cos 7 cells were transfected with either pSV27-C40Apa1 or pSV27-C40Apa1 and pSVHBsAg. Culture fluids were harvested 5 days post transfection. VLPs (prep as above) were examined by western blot

20   (Figure 21).

Virus-like particle vaccination results

Two groups of 3 mice were vaccinated with the virus-like particles, composed of 140 amino acids of HCV core fused with the 19 amino acid tail of HDAg, and enveloped by HBsAg. The particles were prepared by co-transfection of COS7

25   cells using DOTAP. The cell culture supernatant 5 days post transfection was then centrifuged over a 20% sucrose cushion and the particles resuspended in

PBS 100µl of this preparation was injected by the intraperitoneal route, and 2 weeks later the mice were boosted.

After a further 2 weeks, the mice were sacrificed, the spleens removed and the cells stimulated and expanded *in vitro* with specific peptide. To determine the
5   level of cytotoxic T cell activity, control P815 cells or peptide pulsed P815 cells were incubated with the expanded effector cells in a classical ^{51}Cr release assay. The results are shown in figure 22; the level of background killing was high (approximately 40%), but there was a clear increase in cell killing in the HBsAg- and the HCV core-peptide pulsed cells. At an effector:target ratio of
10   100:1, 70.5% and 67% of the cells respectively were killed. Thus, there was a low but consistent CTL response to both HCV core peptides (amino acids 129-140 and amino acids 132-140 respectively) and to the HBsAg peptide (amino acids 28-39). The sequence of the peptides used in these studies were:

HCV core amino acids	129 – 140	GFADLMGYIPLV;
HCV core amino acids	132 – 140	DLMGYIPLV;
HBsAg amino acids	28 – 39	IPQSLDSWWTSL.

All the references cited herein, are hereby incorporated in their entirety by reference. Further while this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill that
20   variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein.

Sequence listings

(1)   **SEQ ID NO: 1**

```
              10            20            30            40
               *             *             *             *
    ATG AGT ACT AAC CCT AAA CCC CAA AGA AAA ACC AAA CGT AAC ACC AAC
    TAC TCA TAG TTC GGA TTT GGG GTT TCT TTT TGG TTT GCA TTG TGG TTG
    Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn

              50            60            70            80            90
               *             *             *             *             *
    CGC CGT CCA CAG GAC GTC AAG TTC CCG GGC GGT GGT CAG ATC GTT GGT
    GCG GCA GGT GTC CTG CAG TTC AAG GGC CCG CCA CCA GTC TAG CAA CCA
    Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly

             100           110           120           130           140
               *             *             *             *             *
    GGA GTT TAC CTG TTG CCG CGC AGG GGC CCC AGG TTG GGT GTG CGC GCG
    CCT CAA ATG GAC AAC GGC GCG TCC CCG GGG TCC AAC CCA CAC GCG CGC
    Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala

             150           160           170           180           190
               *             *             *             *             *
    CTC AGG AAG ACT TCC GAG CGG TCG CAA CCT CGT GGA AGG CGA CAA CCT
    GAG TCC TTC TGA AGG CTC GCC AGC GTT GGA GCA CCT TCC GCT GTT GGA
    Leu Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro

             200           210           220           230           240
               *             *             *             *             *
    ATC CCC AAG GCT CGC CGA CCC GAG GGC AGG GCC TGG GCT CAG CCC GGG
    TAG GGG TTC CGA GCG GCT GGG CTC CCG TCC CGG ACC CGA GTC GGG CCC
    Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Ala Trp Ala Gln Pro Gly

             250           260           270           280
               *             *             *             *
    TAC CCT TGG CCC CTC TAT GGC AAT GAG GGC ATG GGG TGG GCA GGA TGG
    ATG GGA ACC GGG GAG ATA CCG TTA CTC CCG TAC CCC ACC CGT CCT ACC
    Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Met Gly Trp Ala Gly Trp

    290           300           310           320           330
      *             *             *             *             *
    CTC CTG TCA CCC CGT GGT TCT CGG CCT AGT TGG GGC CCC TCA GAC CCC
    GAG GAC AGT GGG GCA CCA AGA GCC GGA TCA ACC CCG GGG AGT CTG GGG
    Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Ser Asp Pro

             340           350           360           370           380
               *             *             *             *             *
    CGG CGT AGG TCG CGT AAT TTG GGT GGA TCC TGG GAT ATA CTC TTC CCA
    GCC GCA TCC AGC GCA TTA AAC CCA CCT AGG ACC CTA TAT GAG AAG GGT
    Arg Arg Arg Ser Arg Asn Leu Gly Gly Ser Trp Asp Ile Leu Phe Pro

             390           400           410           420
               *             *             *             *
    GCC GAT CCG CCC TTT TCT CCC CAG AGT TGT CGA CCC CAG TGA
    CGG CTA GGC GGG AAA AGA GGG GTC TCA ACA GCT GGG GTC ACT
    Ala Asp Pro Pro Phe Ser Pro Gln Ser Cys Arg Pro Gln ***
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(2) **SEQ ID NO: 2**

```
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            *           *           *           *
ATG AGT ACT AAC CCT AAA CCC CAA AGA AAA ACC AAA CGT AAC ACC AAC
TAC TCA TAG TTC GGA TTT GGG GTT TCT TTT TGG TTT GCA TTG TGG TTG
Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn

       50          60          70          80          90
       *           *           *           *           *
CGC CGT CCA CAG GAC GTC AAG TTC CCG GGC GGT GGT CAG ATC GTT GGT
GCG GCA GGT GTC CTG CAG TTC AAG GGC CCG CCA CCA GTC TAG CAA CCA
Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly

       100         110         120         130         140
       *           *           *           *           *
GGA GTT TAC CTG TTG CCG CGC AGG GGC CCC AGG TTG GGT GTG CGC GCG
CCT CAA ATG GAC AAC GGC GCG TCC CCG GGG TCC AAC CCA CAC GCG CGC
Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala

       150         160         170         180         190
       *           *           *           *           *
CTC AGG AAG ACT TCC GAG CGG TCG CAA CCT CGT GGA AGG CGA CAA CCT
GAG TCC TTC TGA AGG CTC GCC AGC GTT GGA GCA CCT TCC GCT GTT GGA
Leu Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro

       200         210         220         230         240
       *           *           *           *           *
ATC CCC AAG GCT CGC CGA CCC GAG GGC AGG GCC TGG GCT CAG CCC GGG
TAG GGG TTC CGA GCG GCT GGG CTC CCG TCC CGG ACC CGA GTC GGG CCC
Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Ala Trp Ala Gln Pro Gly

       250         260         270         280
       *           *           *           *
TAC CCT TGG CCC CTC TAT GGC AAT GAG GGC ATG GGG TGG GCA GGA TGG
ATG GGA ACC GGG GAG ATA CCG TTA CTC CCG TAC CCC ACC CGT CCT ACC
Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Met Gly Trp Ala Gly Trp

290         300         310         320         330
*           *           *           *           *
CTC CTG TCA CCC CGT GGT TCT CGG CCT AGT TGG GGC CCC TCA GAC CCC
GAG GAC AGT GGG GCA CCA AGA GCC GGA TCA ACC CCG GGG AGT CTG GGG
Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Ser Asp Pro

       340         350         360         370         380
       *           *           *           *           *
CGG CGT AGG TCG CGT AAT TTG GGT AAG GTC ATC GAT ACC CTT ACA TGC
GCC GCA TCC AGC GCA TTA AAC CCA TTC CAG TAG CTA TGG GAA TGT ACG
Arg Arg Arg Ser Arg Asn Leu Gly Lys Val Ile Asp Thr Leu Thr Cys

       390         400         410         420         430
       *           *           *           *           *
GGC TTC GCC GAC CTC ATG GGG TAC ATT CCG CTC GTC GGA TCC TGG GAT
CCG AAG CGG CTG GAG TAC CCC ATG TAA GGC GAG CAG CCT AGG ACC CTA
Gly Phe Ala Asp Leu Met Gly Tyr Ile Pro Leu Val Gly Ser Trp Asp

       440         450         460         470         480
       *           *           *           *           *
ATA CTC TTC CCA GCC GAT CCG CCC TTT TCT CCC CAG AGT TGT CGA CCC
TAT GAG AAG GGT CGG CTA GGC GGG AAA AGA GGG GTC TCA ACA GCT GGG
Ile Leu Phe Pro Ala Asp Pro Pro Phe Ser Pro Gln Ser Cys Arg Pro

CAG TGA
GTC ACT
Gln ***
```

(3)   **SEQ ID NO: 3**

```
              10          20          30          40
               *           *           *           *
     ATG AGT ACT AAC CCT AAA CCC CAA AGA AAA ACC AAA CGT AAC ACC AAC
     TAC TCA TAG TTC GGA TTT GGG GTT TCT TTT TGG TTT GCA TTG TGG TTG
     Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn

     50          60          70          80          90
      *           *           *           *           *
     CGC CGT CCA CAG GAC GTC AAG TTC CCG GGC GGT GGT CAG ATC GTT GGT
     GCG GCA GGT GTC CTG CAG TTC AAG GGC CCG CCA CCA GTC TAG CAA CCA
     Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly

     100         110         120         130         140
      *           *           *           *           *
     GGA GTT TAC CTG TTG CCG CGC AGG GGC CCC AGG TTG GGT GTG CGC GCG
     CCT CAA ATG GAC AAC GGC GCG TCC CCG GGG TCC AAC CCA CAC GCG CGC
     Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala

     150         160         170         180         190
      *           *           *           *           *
     CTC AGG AAG ACT TCC GAG CGG TCG CAA CCT CGT GGA AGG CGA CAA CCT
     GAG TCC TTC TGA AGG CTC GCC AGC GTT GGA GCA CCT TCC GCT GTT GGA
     Leu Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro

          200         210         220         230         240
           *           *           *           *           *
     ATC CCC AAG GCT CGC CGA CCC GAG GGC AGG GCC TGG GCT CAG CCC GGG
     TAG GGG TTC CGA GCG GCT GGG CTC CCG TCC CGG ACC CGA GTC GGG CCC
     Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Ala Trp Ala Gln Pro Gly

          250         260         270         280
           *           *           *           *
     TAC CCT TGG CCC CTC TAT GGC AAT GAG GGC ATG GGG TGG GCA GGA TGG
     ATG GGA ACC GGG GAG ATA CCG TTA CTC CCG TAC CCC ACC CGT CCT ACC
     Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Met Gly Trp Ala Gly Trp

     290         300         310         320         330
      *           *           *           *           *
     CTC CTG TCA CCC CGT GGT TCT CGG CCT AGT TGG GGC CCC TCA GAC CCC
     GAG GAC AGT GGG GCA CCA AGA GCC GGA TCA ACC CCG GGG AGT CTG GGG
     Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Ser Asp Pro

     340         350         360         370         380
      *           *           *           *           *
     CGG CGT AGG TCG CGT AAT TTG GGT AAG GTC ATC GAT ACC CTT ACA TGC
     GCC GCA TCC AGC GCA TTA AAC CCA TTC CAG TAG CTA TGG GAA TGT ACG
     Arg Arg Arg Ser Arg Asn Leu Gly Lys Val Ile Asp Thr Leu Thr Cys

     390         400         410         420         430
      *           *           *           *           *
     GGC TTC GCC GAC CTC ATG GGG TAC ATT CCG CTC GTC GGC GCC CCT CTA
     CCG AAG CGG CTG GAG TAC CCC ATG TAA GGC GAG CAG CCG CGG GGA GAT
     Gly Phe Ala Asp Leu Met Gly Tyr Ile Pro Leu Val Gly Ala Pro Leu
```

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                   440              450              460              470              480
                    *                *                *                *                *
        GGG GGC GCC GCC AGG GCC CTG GCG CAT GGC GTC CGG GTT CTG GAG GAC
5       CCC CCG CGG CGG TCC CGG GAC CGC GTA CCG CAG GCC CAA GAC CTC CTG
        Gly Gly Ala Ala Arg Ala Leu Ala His Gly Val Arg Val Leu Glu Asp

                   490              500              510              520
                    *                *                *                *
10      GGC GGA TCC TGG GAT ATA CTC TTC CCA GCC GAT CCG CCC TTT TCT CCC
        CCG CCT AGG ACC CTA TAT GAG AAG GGT CGG CTA GGC GGG AAA AGA GGG
        Gly Gly Ser Trp Asp Ile Leu Phe Pro Ala Asp Pro Pro Phe Ser Pro

        530              540
         *                *
15      CAG AGT TGT CGA CCC CAG TGA
        GTC TCA ACA GCT GGG GTC ACT
        Gln Ser Cys Arg Pro Gln ***
```

THE CLAIMS of the invention are as follows:

1. A virus-like particle for use in the treatment or prevention of at least a microorganism infection wherein said particle comprises: at least a antigenic and/or immunogenic polypeptide or part thereof from the microorganism, fused to at least the last 19 amino acid of the COOH terminal sequence of the large protein from Hepatitis D virus, wherein the fusion protein is at least partially enveloped by Hepatitis B surface antigen.

2. A virus-like particle according to claim 1 wherein the immunogenic polypeptide is capable of eliciting a T cell response.

3. A virus-like particle according to claim 1 wherein the immunogenic polypeptide is capable of eliciting a humoral response.

4. A virus-like particle according to claim 1 wherein the immunogenic polypeptide or part thereof expresses a plurality of epitopes.

5. A virus-like particle according to claim 1 wherein the immunogenic polypeptide or part thereof expresses a plurality of epitopes, which as a whole should be capable of eliciting a humoral response and a T cell response.

6. A virus-like particle according to any one of claims 1, 2, 4 or 5 wherein the immunogenic polypeptide or part thereof expresses a plurality of epitopes wherein at least one of the epitopes is capable of stimulating a cytotoxic T lymphocyte response.

7. A virus-like particle according to any one of the previous claims wherein the immunogenic polypeptide is derived from hepatitis C virus.

8.   A virus-like particle according to claim 7 wherein the immunogenic polypeptide is derived from HCV core protein.

9.   A virus-like particle according to claim 7 wherein the immunogenic polypeptide is derived from HCV NS3 protein.

10.   A virus-like particle according to claim 7 wherein the immunogenic polypeptide is derived from amino acids 1 to 191 of the HCV core protein.

11.   A virus-like particle according to claim 8 wherein the immunogenic polypeptide is between about 120 and 160 amino acids in length.

12.   A virus-like particle according to claim 1 wherein the fusion protein is selected from any one of SEQ ID NO 1, SEQ ID NO 2 or SEQ ID NO 3.

13.   A virus-like particle according to claim 1 wherein the fusion protein is substantially homologous to any one of SEQ ID NO 1, SEQ ID NO 2 or SEQ ID NO 3.

14.   A virus like particle according to any one of the preceding claims wherein the entire amino acid sequence of L-HDAg is used in the fusion protein.

15.   A method for producing virus-like particles containing an antigenic and or immunogenic polypeptide or part thereof comprising the step of: incubating host cells transformed with an expression vector which includes a nucleotide sequence encoding a fusion polypeptide containing the antigenic and or immunogenic polypeptide or part thereof and at least the last 19 amino acid of the COOH terminal sequence of the large protein from Hepatitis D virus; in the presence of HBsAg, under conditions which allow expression and packaging of said fusion polypeptide.

16.   A method according to claim 14 wherein HBsAg is expressed in the same host cells as the fusion polypeptide.

17. A method according to any one of claims 15 or 16 wherein the entire amino acid sequence of L-HDAg is used in the fusion protein.

18. A therapeutic for use in the treatment of a patient suffering from at least a microorganism infection, said therapeutic comprising: virus like particles according to any one of claims 1 to 13 in combination with a pharmaceutically acceptable carrier.

19. A therapeutic for aiding in the prevention of at least a microorganism infection in a patient, said therapeutic comprising: virus like particles according to any one of claims 1 to 13 in combination with a pharmaceutically acceptable carrier.

20. A therapeutic according any one of claims 18 or 19 wherein the entire amino acid sequence of L-HDAg is used in the fusion protein.

Fig. 1.
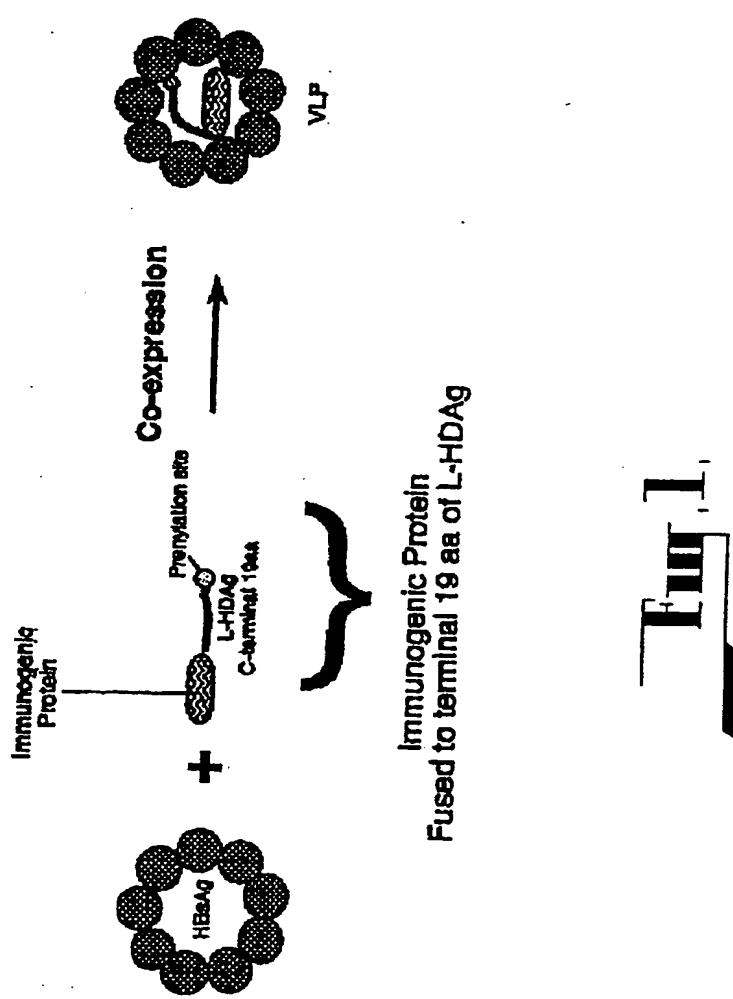
Fig. 2.

```
          10              20              30              40
           *               *               *               *
NNG AGG TCT CGT AGA CCG TGC ACC ATG AGC ACG AAT CCT AAA CCT CAA
NNC TCC AGA GCA TCT GGC ACG TGG TAC TCG TGC TTA GGA TTT GGA GTT
Xxx Arg Ser Arg Arg Pro Cys Thr Met Ser Thr Asn Pro Lys Pro Gln

  50              60              70              80              90
   *               *               *               *               *
AGA AAA ACC AAA CGT AAC ACC AAC CGC CGT CCA CAG GAC GTC AAG TTC
TCT TTT TGG TTT GCA TTG TGG TTG GCG GCA GGT GTC CTG CAG TTC AAG
Arg Lys Thr Lys Arg Asn Thr Asn Arg Arg Pro Gln Asp Val Lys Phe

     100             110             120             130             140
      *               *               *               *               *
CCG GGC GGT GGT CAG ATC GTT GGT GGA GTT TAC CTG TTG CCG CGC AGG
GGC CCG CCA CCA GTC TAG CAA CCA CCT CAA ATG GAC AAC GGC GCG TCC
Pro Gly Gly Gly Gln Ile Val Gly Gly Val Tyr Leu Leu Pro Arg Arg

        150             160             170             180             190
         *               *               *               *               *
GGC CCC AGG TTG GGT GTG CGC GCG CTC AGG AAG ACT TCC GAG CGG TCG
CCG GGG TCC AAC CCA CAC GCG CGC GAG TCC TTC TGA AGG CTC GCC AGC
Gly Pro Arg Leu Gly Val Arg Ala Leu Arg Lys Thr Ser Glu Arg Ser

           200             210             220             230             240
            *               *               *               *               *
CAA CCT CGT GGA AGG CGA CAA CCT ATC CCC AAG GCT CGC CGA CCC GAG
GTT GGA GCA CCT TCC GCT GTT GGA TAG GGG TTC CGA GCG GCT GGG CTC
Gln Pro Arg Gly Arg Arg Gln Pro Ile Pro Lys Ala Arg Arg Pro Glu

              250             260             270             280
               *               *               *               *
GGC AGG GCC TGG GCT CAG CCC GGG TAC CCT TGG CCC CTC TAT GGC AAT
CCG TCC CGG ACC CGA GTC GGG CCC ATG GGA ACC GGG GAG ATA CCG TTA
Gly Arg Ala Trp Ala Gln Pro Gly Tyr Pro Trp Pro Leu Tyr Gly Asn

290             300             310             320             330
 *               *               *               *               *
GAG GGC ATG GGG TGG GCA GGA TGG CTC CTG TCA CCC CGT GGT TCT CGG
CTC CCG TAC CCC ACC CGT CCT ACC GAG GAC AGT GGG GCA CCA AGA GCC
Glu Gly Met Gly Trp Ala Gly Trp Leu Leu Ser Pro Arg Gly Ser Arg

   340             350             360             370             380
    *               *               *               *               *
CCT AGT TGG GGC CCC TCA GAC CCC CGG CGT AGG TCG CGT AAT TTG GGT
GGA TCA ACC CCG GGG AGT CTG GGG GCC GCA TCC AGC GCA TTA AAC CCA
Pro Ser Trp Gly Pro Ser Asp Pro Arg Arg Arg Ser Arg Asn Leu Gly

      390             400             410             420             430
       *               *               *               *               *
AAG GTC ATC GAT ACC CTT ACA TGC GGC TTC GCC GAC CTC ATG GGG TAC
TTC CAG TAG CTA TGG GAA TGT ACG CCG AAG CGG CTG GAG TAC CCC ATG
Lys Val Ile Asp Thr Leu Thr Cys Gly Phe Ala Asp Leu Met Gly Tyr
```

```
          440           450           460           470           480
           *             *             *             *             *
ATT CCG CTC GTC GGC GCC CCT CTA GGG GGC GCC GCC AGG GCC CTG GCG
TAA GGC GAG CAG CCG CGG GGA GAT CCC CCG CGG CGG TCC CGG GAC CGC
Ile Pro Leu Val Gly Ala Pro Leu Gly Gly Ala Ala Arg Ala Leu Ala


          490           500           510           520
           *             *             *             *
CAT GGC GTC CGG GTT CTG GAG GAC GGC GTG AAC TAT GCA ACA GGG AGC
GTA CCG CAG GCC CAA GAC CTC CTG CCG CAC TTG ATA CGT TGT CCC TCG
His Gly Val Arg Val Leu Glu Asp Gly Val Asn Tyr Ala Thr Gly Ser


530
 *
ACC GG
TGG CC
Thr Gly
```

Fig.3.

```
         10                20                30                40
          *                 *                 *                 *
NTG GCA TGG GAT ATG ATG ATG AAC TGG TCA CCC ACA GCA GCC CTA GTC
NAC CGT ACC CTA TAC TAC TAC TTG ACC AGT GGG TGT CGT CGG GAT CAG
Xxx Ala Trp Asp Met Met Met Asn Trp Ser Pro Thr Ala Ala Leu Val

 50                60                70                80                90
  *                 *                 *                 *                 *
GTA TCG CAG TTA CTC CGG ATC CCA CAA GCT ATC GTG GAT ATG GTG GCG
CAT AGC GTC AAT GAG GCC TAG GGT GTT CGA TAG CAC CTA TAC CAC CGC
Val Ser Gln Leu Leu Arg Ile Pro Gln Ala Ile Val Asp Met Val Ala

       100               110               120               130               140
        *                 *                 *                 *                 *
GGG GCC CAC TGG GGA GTC CTG GCG GGC CTC GCC TAC TAT TCC ATG GTG
CCC CGG GTG ACC CCT CAG GAC CGC CCG GAG CGG ATG ATA AGG TAC CAC
Gly Ala His Trp Gly Val Leu Ala Gly Leu Ala Tyr Tyr Ser Met Val

       150               160               170               180               190
        *                 *                 *                 *                 *
GGG AAC TGG GCT AAG GTT TTG ATT GTG ATG CTA CTC TTT GAC GGC GTT
CCC TTG ACC CGA TTC CAA AAC TAA CAC TAC GAT GAG AAA CTG CCG CAA
Gly Asn Trp Ala Lys Val Leu Ile Val Met Leu Leu Phe Asp Gly Val

         200               210               220               230               240
          *                 *                 *                 *                 *
GAC GGG GAC ACC CAC ACG ACG GGG GGG GTG GCG GGC CGC GAC ACG CTG
CTG CCC CTG TGG GTG TGC TGC CCC CCC CAC CGC CCG GCG CTG TGC GAC
Asp Gly Asp Thr His Thr Thr Gly Gly Val Ala Gly Arg Asp Thr Leu

         250               260               270               280
          *                 *                 *                 *
CGC TTC ACG GGG TTC TTT TCA TTG GGG CCG AAA CAA AAG ATC CAG CTT
GCG AAG TGC CCC AAG AAA AGT AAC CCC GGC TTT GTT TTC TAG GTC GAA
Arg Phe Thr Gly Phe Phe Ser Leu Gly Pro Lys Gln Lys Ile Gln Leu

290               300               310               320               330
  *                 *                 *                 *                 *
GTA AAC ACC AAC GGC AGC TGG CAC ATC AAC AGG ACT GCC CTG AAC TGC
CAT TTG TGG TTG CCG TCG ACC GTG TAG TTG TCC TGA CGG GAC TTG ACG
Val Asn Thr Asn Gly Ser Trp His Ile Asn Arg Thr Ala Leu Asn Cys

   340               350               360               370               380
    *                 *                 *                 *                 *
AAT GAC TCC CTC AAA ACT GGG TGG CTC GCC GCG CTG TTC TAC ACA CAC
TTA CTG AGG GAG TTT TGA CCC ACC GAG CGG CGC GAC AAG ATG TGT GTG
Asn Asp Ser Leu Lys Thr Gly Trp Leu Ala Ala Leu Phe Tyr Thr His

   390               400               410               420               430
    *                 *                 *                 *                 *
AGC TTC AAC GCG TCT GGA TGC CCA GAG CGG ATG GCC AGC TGC CAC CCC
TCG AAG TTG CGC AGA CCT ACG GGT CTC GCC TAC CGG TCG ACG GTG GGG
Ser Phe Asn Ala Ser Gly Cys Pro Glu Arg Met Ala Ser Cys His Pro
```

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              440          450          460          470          480
               *            *            *            *            *
     ATC GAC GAG TTC GCT CAG GGG TGG GGT CCC ATT ACT TAC GCT GAA CAT
     TAG CTG CTC AAG CGA GTC CCC ACC CCA GGG TAA TGA ATG CGA CTT GTA
     Ile Asp Glu Phe Ala Gln Gly Trp Gly Pro Ile Thr Tyr Ala Glu His


              490          500          510          520
               *            *            *            *
     AGC AGC TCG GAC CAG AGG CCC TAC TGT TGG CAC TAC GCA CCT CAG CCG
     TCG TCG AGC CTG GTC TCC GGG ATG ACA ACC GTG ATG CGT GGA GTC GGC
     Ser Ser Ser Asp Gln Arg Pro Tyr Cys Trp His Tyr Ala Pro Gln Pro


     530          540          550          560          570
      *            *            *            *            *
     TGC GGT ATT GTA CCC GCG TCG GAG GTG TGT GGT CCA GTG TAT TGC TTC
     ACG CCA TAA CAT GGG CGC AGC CTC CAC ACA CCA GGT CAC ATA ACG AAG
     Cys Gly Ile Val Pro Ala Ser Glu Val Cys Gly Pro Val Tyr Cys Phe


          580          590          600          610
           *            *            *            *
     ACC CCA AGC CCT GTT GTG GTG GGG ACG ACC GAG CAC CGG
     TGG GGT TCG GGA CAA CAC CAC CCC TGC TGG CTC GTG GCC
     Thr Pro Ser Pro Val Val Val Gly Thr Thr Glu His Arg
```

Fig. 4.

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        10              20              30              40
         *               *               *               *
GGT AAG GTC ATC GAT ACC CTT ACA TGC GGC TTC GCC GAC CTC ATG GGG
CCA TTC CAG TAG CTA TGG GAA TGT ACG CCG AAG CGG CTG GAG TAC CCC
Gly Lys Val Ile Asp Thr Leu Thr Cys Gly Phe Ala Asp Leu Met Gly

  50              60              70              80              90
   *               *               *               *               *
TAC ATT CCG CTC GTC GGC GCC CCT CTA GGG GGC GCC GCC AGG GCC CTG
ATG TAA GGC GAG CAG CCG CGG GGA GAT CCC CCG CGG CGG TCC CGG GAC
Tyr Ile Pro Leu Val Gly Ala Pro Leu Gly Gly Ala Ala Arg Ala Leu

    100             110             120             130             140
     *               *               *               *               *
GCG CAT GGC GTC CGG GTT CTG GAG GAC GGC GTG AAC TAT GCA ACA GGG
CGC GTA CCG CAG GCC CAA GAC CTC CTG CCG CAC TTG ATA CGT TGT CCC
Ala His Gly Val Arg Val Leu Glu Asp Gly Val Asn Tyr Ala Thr Gly

      150             160             170             180             190
       *               *               *               *               *
AAT TTG CCC GGT TGC TCT TTC TCT ATC TTC CTC TTG GGT TTG CTG TCT
TTA AAC GGG CCA ACG AGA AAG AGA TAG AAG GAG AAC CCA AAC GAC AGA
Asn Leu Pro Gly Cys Ser Phe Ser Ile Phe Leu Leu Gly Leu Leu Ser

        200             210             220             230             240
         *               *               *               *               *
TGT TTG ACC ATC CCA GCT TCC GCT TAT GAA GTG CGC AAC GTG TCC GGG
ACA AAC TGG TAG GGT CGA AGG CGA ATA CTT CAC GCG TTG CAC AGG CCC
Cys Leu Thr Ile Pro Ala Ser Ala Tyr Glu Val Arg Asn Val Ser Gly

          250             260             270             280
           *               *               *               *
GTG TAC CAT GTC ACG AAC GAC TGC TCC AAC GCA AGC ATT GTG TAT GAG
CAC ATG GTA CAG TGC TTG CTG ACG AGG TTG CGT TCG TAA CAC ATA CTC
Val Tyr His Val Thr Asn Asp Cys Ser Asn Ala Ser Ile Val Tyr Glu

290             300             310             320             330
 *               *               *               *               *
GCA GCG GAC ATG ATC ATG CAC GTC CCC GGG TGC GTG CCC TGC GTT CGG
CGT CGC CTG TAC TAG TAC GTG CAG GGG CCC ACG CAC GGG ACG CAA GCC
Ala Ala Asp Met Ile Met His Val Pro Gly Cys Val Pro Cys Val Arg

    340             350             360             370             380
     *               *               *               *               *
GTG GAC AAC TCC TCC CGT TGC TGG GTA GCG CTC ACC CCC ACG CTT GCG
CAC CTG TTG AGG AGG GCA ACG ACC CAT CGC GAG TGG GGG TGC GAA CGC
Val Asp Asn Ser Ser Arg Cys Trp Val Ala Leu Thr Pro Thr Leu Ala

      390             400             410             420             430
       *               *               *               *               *
GCC AGG AAC GCT AGC GTC CCT ACT ACG GCA ATA CGA CGC CAC GTC GAT
CGG TCC TTG CGA TCG CAG GGA TGA TGC CGT TAT GCT GCG GTG CAG CTA
Ala Arg Asn Ala Ser Val Pro Thr Thr Ala Ile Arg Arg His Val Asp
```

17:32:56

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                440              450              460              470              48C
                 *                *                *                *                *
       TTG CTC GTT GGG GCG GCT ACT TTC TGT TCC GCT ATG TAC GTG GGG GAT
       AAC GAG CAA CCC CGC CGA TGA AAG ACA AGG CGA TAC ATG CAC CCC CTA
       Leu Leu Val Gly Ala Ala Thr Phe Cys Ser Ala Met Tyr Val Gly Asp

                    490              500              510              520
                     *                *                *                *
       CTC TGC GGA TCT GTT TTC CTC GTC GCC CAG CTG TTC ACC TTC TCG CCC
       GAG ACG CCT AGA CAA AAG GAG CAG CGG GTC GAC AAG TGG AAG AGC GGG
       Leu Cys Gly Ser Val Phe Leu Val Ala Gln Leu Phe Thr Phe Ser Pro

       530              540              550              560              570
        *                *                *                *                *
       CGC CGG CAT GAG ACG GTA CAG GAC TGC AAT TGT TCA ATC TAT CCC GGC
       GCG GCC GTA CTC TGC CAT GTC CTG ACG TTA ACA AGT TAG ATA GGG CCG
       Arg Arg His Glu Thr Val Gln Asp Cys Asn Cys Ser Ile Tyr Pro Gly

           580              590              600              610              620
            *                *                *                *                *
       CAC ATA ACG GGT CAC CGC ATG GCT TGG GAT ATG ATG ATG AAC TGG TCA
       GTG TAT TGC CCA GTG GCG TAC CGA ACC CTA TAC TAC TAC TTG ACC AGT
       His Ile Thr Gly His Arg Met Ala Trp Asp Met Met Met Asn Trp Ser

           630              640              650              660              670
            *                *                *                *                *
       CCC ACA GCA GCC CTA GTC GTA TCG CAG TTA CTC CGG ATC CCA CAA GAA A
       GGG TGT CGT CGG GAT CAG CAT AGC GTC AAT GAG GCC TAG GGT GTT CTT T
       Pro Thr Ala Ala Leu Val Val Ser Gln Leu Leu Arg Ile Pro Gln Glu
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Fig. 5.
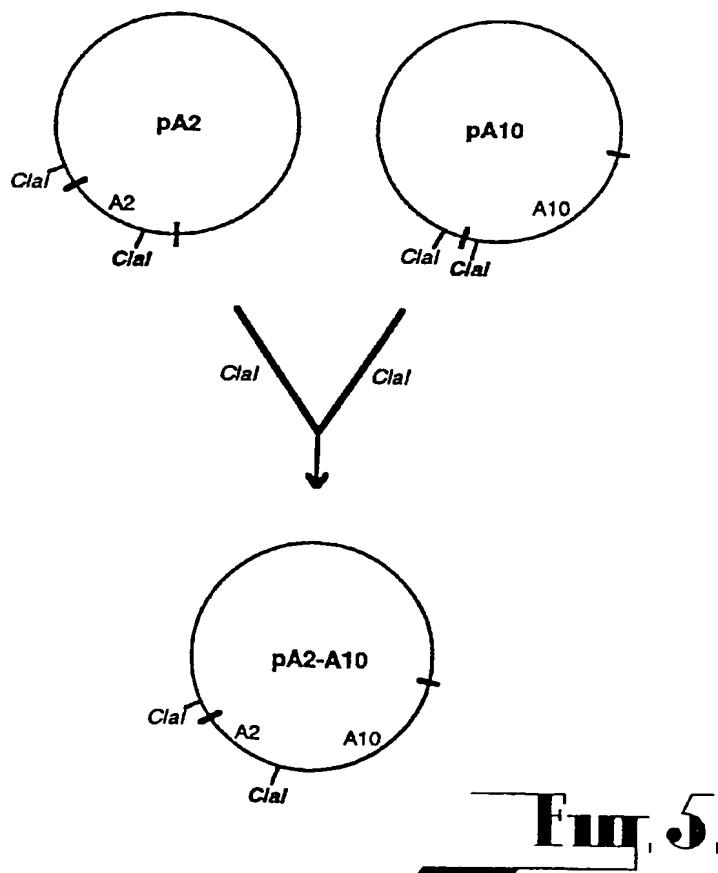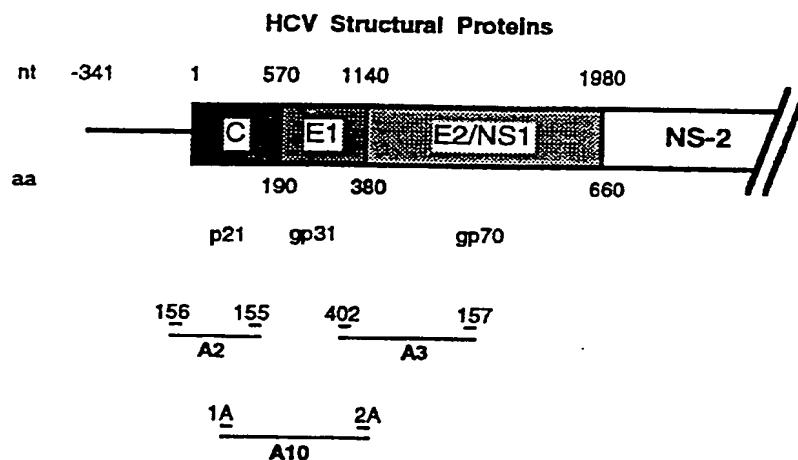
Fig. 6.

```
          10              20              30              40
          *               *               *               *
ATG GAG AAC ATC ACA TCA GGA TTC CTA GGA CCC CTT CTC GTG TTA CAG
TAC CTC TTG TAG TGT AGT CCT AAG GAT CCT GGG GAA GAG CAC AAT GTC
Met Glu Asn Ile Thr Ser Gly Phe Leu Gly Pro Leu Leu Val Leu Gln

          50              60              70              80              90
          *               *               *               *               *
GCG GGG TTT TTC TTG TTG ACA AGA ATC CTC ACA ATA CCG CAG AGT CTA
CGC CCC AAA AAG AAC AAC TGT TCT TAG GAG TGT TAT GGC GTC TCA GAT
Ala Gly Phe Phe Leu Leu Thr Arg Ile Leu Thr Ile Pro Gln Ser Leu

          100             110             120             130             140
          *               *               *               *               *
GAA TCG TGG TGG ACT TCT CTC AAT TTT TTA GGG GGA AAT ACC GTG TGT
CTT AGC ACC ACC TGA AGA GAG TTA AAA AAT CCC CCT TTA TGG CAC ACA
Glu Ser Trp Trp Thr Ser Leu Asn Phe Leu Gly Gly Asn Thr Val Cys

          150             160             170             180             190
          *               *               *               *               *
CTT GGC CAA AAT TCG CAG TCC CCA ACC TCC AAT CAC TCA CCA ACC TCC
GAA CCG GTT TTA AGC GTC AGG GGT TGG AGG TTA GTG AGT GGT TGG AGG
Leu Gly Gln Asn Ser Gln Ser Pro Thr Ser Asn His Ser Pro Thr Ser

          200             210             220             230             240
          *               *               *               *               *
TGT CCT CCA ACT TGT CCT GGT TAT CGC TGG ATG TGT CTG CGG CGT TTT
ACA GGA GGT TGA ACA GGA CCA ATA GCG ACC TAC ACA GAC GCC GCA AAA
Cys Pro Pro Thr Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe

          250             260             270             280
          *               *               *               *
ATC ATC TTC CTC TTC ATC CTG CTG CTA TGC CTC ATC TTC TTG TTG GTT
TAG TAG AAG GAG AAG TAG GAC GAC GAT ACG GAG TAG AAG AAC AAC CAA
Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val

290             300             310             320             330
  *               *               *               *               *
CTT CTG GAC TAT CAA GGT ATG TTG CCC GTT TGT CCT CTA ATT CCA GGA
GAA GAC CTG ATA GTT CCA TAC AAC GGG CAA ACA GGA GAT TAA GGT CCT
Leu Leu Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly

          340             350             360             370             380
          *               *               *               *               *
TCA TCA ACC ACC AGC ACG GGA CCC TGC AGA ACC TGC ACG ACT CCT GCT
AGT AGT TGG TGG TCG TGC CCT GGG ACG TCT TGG ACG TGC TGA GGA CGA
Ser Ser Thr Thr Ser Thr Gly Pro Cys Arg Thr Cys Thr Thr Pro Ala

          390             400             410             420             430
          *               *               *               *               *
CAA GGA ACC TCT ATG TAT CCC TCC TGT TGC TGT ACA AAA CCT TCG GAC
GTT CCT TGG AGA TAC ATA GGG AGG ACA ACG ACA TGT TTT GGA AGC CTG
Gln Gly Thr Ser Met Tyr Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp
```

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         440         450         460         470         480
          *           *           *           *           *
GGA AAC TGC ACC TGT ATT CCC ATC CCA TCA TCC TGG GCT TTC GGA AAA
CCT TTG ACG TGG ACA TAA GGG TAG GGT AGT AGG ACC CGA AAG CCT TTT
Gly Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Gly Lys

              490         500         510         520
               *           *           *           *
TTC CTA TGG GAG TGG GCC TCA GCC CGT TTC TCT TGG CTC AGT TTA CTA
AAG GAT ACC CTC ACC CGG AGT CGG GCA AAG AGA ACC GAG TCA AAT GAT
Phe Leu Trp Glu Trp Ala Ser Ala Arg Phe Ser Trp Leu Ser Leu Leu

530         540         550         560         570
 *           *           *           *           *
GTG CCA TTT GTT CAG TGG TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT
CAC GGT AAA CAA GTC ACC AAG CAT CCC GAA AGG GGG TGA CAA ACC GAA
Val Pro Phe Val Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu

    580         590         600         610         620
     *           *           *           *           *
TCA GTT ATA TGG ATG ATG TGG TAT TGG GGG CCA AGT CTG TAC AGC ATC
AGT CAA TAT ACC TAC TAC ACC ATA ACC CCC GGT TCA GAC ATG TCG TAG
Ser Val Ile Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Ser Ile

    630         640         650         660         670
     *           *           *           *           *
TTG AGT CCC TTT TTA CCG CTG TTA CCA ATT TTC TTT TGT CTT TGG GCA
AAC TCA GGG AAA AAT GGC GAC AAT GGT TAA AAG AAA ACA GAA ACC CGT
Leu Ser Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Ala

    680
     *
TAC ATT TAA
ATG TAA ATT
Tyr Ile ***
```

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Fig.7

```
...........TGG GAT ATA CTC TTC CCA GCC GAT CCG CCC TTT TCT CCC CAG AGT
...........ACC CTA TAT GAG AAG GGT CGG CTA GGC GGG AAA AGA GGG GTC TCA
...........Trp Asp Ile Leu Phe Pro Ala Asp Pro Pro Phe Ser Pro Gln Ser

TGT CGA CCC CAG TGA
ACA GCT GGG GTC ACT
Cys Arg Pro Gln ***
```

Fig. 8.

```
                 10              20              30              40
                  *               *               *               *
L-HDAg 19a   TGG GAT ATA CTC TTC CCA GCC GAT CCG CCC TTT TCT CCC CAG AGT TGT

It HDAg se      590         600         610         620         630
[ 234 ]         .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...


Kos HDAg s      590         600         610         620         630
[ 234 ]         .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...


WC HDAg se      590         600         610         620         630
[ 234 ]         .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...


Taiwan 1 H      ·590        600         610         620         630
[ 228 ]         ... ... T.. ... ... ... ... ... ..T ... ... ... ... ... ... ...


USA 1 HDAg      590         600         610         620         630
[ 222 ]         ... ... ... ... ... ... ... ..C ..T ... ..C ... ... ... ... ...



                 50              60
                  *               *
L-HDAg 19a   CGA CCC CAG TGA

It HDAg se          640
[ 234 ]         ... ... ... ...


Kos HDAg s          640
[ 234 ]         ... ... ... ...


WC HDAg se          640
[ 234 ]         •... ... ... ...


Taiwan 1 H          640
[ 228 ]         ... ... ... ...


USA 1 HDAg          640
[ 222 ]         ... ... ... ...
```

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Fig. 9

Fig. 10

Fig.11.

```
          10              20              30              40
           *               *               *               *
ATG AGC ACG AAT CCT AAA CCT CAA AGA AAA ACC AAA CGT AAC ACC AAC
TAC TCG TGC TTA GGA TTT GGA GTT TCT TTT TGG TTT GCA TTG TGG TTG
Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn

 50              60              70              80              90
  *               *               *               *               *
CGC CGT CCA CAG GAC GTC AAG TTC CCG GGC GGT GGT CAG ATC GTT GGT
GCG GCA GGT GTC CTG CAG TTC AAG GGC CCG CCA CCA GTC TAG CAA CCA
Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly

  100             110             120             130             140
   *               *               *               *               *
GGA GTT TAC CTG TTG CCG CGC AGG GGC CCC AGG TTG GGT GTG CGC GCG
CCT CAA ATG GAC AAC GGC GCG TCC CCG GGG TCC AAC CCA CAC GCG CGC
Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala

     150             160             170             180             190
      *               *               *               *               *
CTC AGG AAG ACT TCC GAG CGG TCG CAA CCT CGT GGA AGG CGA CAA CCT
GAG TCC TTC TGA AGG CTC GCC AGC GTT GGA GCA CCT TCC GCT GTT GGA
Leu Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro

        200             210             220             230             240
         *               *               *               *               *
ATC CCC AAG GCT CGC CGA CCC GAG GGC AGG GCC TGG GCT CAG CCC GGG
TAG GGG TTC CGA GCG GCT GGG CTC CCG TCC CGG ACC CGA GTC GGG CCC
Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Ala Trp Ala Gln Pro Gly

           250             260             270             280
            *               *               *               *
TAC CCT TGG CCC CTC TAT GGC AAT GAG GGC ATG GGG TGG GCA GGA TGG
ATG GGA ACC GGG GAG ATA CCG TTA CTC CCG TAC CCC ACC CGT CCT ACC
Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Met Gly Trp Ala Gly Trp

290             300             310             320             330
  *               *               *               *               *
CTC CTG TCA CCC CGT GGT TCT CGG CCT AGT TGG GGC CCC TCA GAC CCC
GAG GAC AGT GGG GCA CCA AGA GCC GGA TCA ACC CCG GGG AGT CTG GGG
Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Ser Asp Pro

   340             350             360             370             380
    *               *               *               *               *
CGG CGT AGG TCG CGT AAT TTG GGT GGA TCC TGG GAT ATA CTC TTC CCA
GCC GCA TCC AGC GCA TTA AAC CCA CCT AGG ACC CTA TAT GAG AAG GGT
Arg Arg Arg Ser Arg Asn Leu Gly Gly Ser Trp Asp Ile Leu Phe Pro

      390             400             410             420
       *               *               *               *
GCC GAT CCG CCC TTT TCT CCC CAG AGT TGT CGA CCC CAG TGA
CGG CTA GGC GGG AAA AGA GGG GTC TCA ACA GCT GGG GTC ACT
Ala Asp Pro Pro Phe Ser Pro Gln Ser Cys Arg Pro Gln ***
```

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Fig.12.

```
              10            20            30            40
               *             *             *             *
     ATG AGT ACT AAC CCT AAA CCC CAA AGA AAA ACC AAA CGT AAC ACC AAC
     TAC TCA TAG TTC GGA TTT GGG GTT TCT TTT TGG TTT GCA TTG TGG TTG
     Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn

      50            60            70            80            90
       *             *             *             *             *
     CGC CGT CCA CAG GAC GTC AAG TTC CCG GGC GGT GGT CAG ATC GTT GGT
     GCG GCA GGT GTC CTG CAG TTC AAG GGC CCG CCA CCA GTC TAG CAA CCA
     Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly

        100           110           120           130           140
         *             *             *             *             *
     GGA GTT TAC CTG TTG CCG CGC AGG GGC CCC AGG TTG GGT GTG CGC GCG
     CCT CAA ATG GAC AAC GGC GCG TCC CCG GGG TCC AAC CCA CAC GCG CGC
     Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala

        150           160           170           180           190
         *             *             *             *             *
     CTC AGG AAG ACT TCC GAG CGG TCG CAA CCT CGT GGA AGG CGA CAA CCT
     GAG TCC TTC TGA AGG CTC GCC AGC GTT GGA GCA CCT TCC GCT GTT GGA
     Leu Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro

          200           210           220           230           240
           *             *             *             *             *
     ATC CCC AAG GCT CGC CGA CCC GAG GGC AGG GCC TGG GCT CAG CCC GGG
     TAG GGG TTC CGA GCG GCT GGG CTC CCG TCC CGG ACC CGA GTC GGG CCC
     Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Ala Trp Ala Gln Pro Gly

            250           260           270           280
             *             *             *             *
     TAC CCT TGG CCC CTC TAT GGC AAT GAG GGC ATG GGG TGG GCA GGA TGG
     ATG GGA ACC GGG GAG ATA CCG TTA CTC CCG TAC CCC ACC CGT CCT ACC
     Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Met Gly Trp Ala Gly Trp

     290           300           310           320           330
      *             *             *             *             *
     CTC CTG TCA CCC CGT GGT TCT CGG CCT AGT TGG GGC CCC TCA GAC CCC
     GAG GAC AGT GGG GCA CCA AGA GCC GGA TCA ACC CCG GGG AGT CTG GGG
     Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Ser Asp Pro

       340           350           360           370           380
        *             *             *             *             *
     CGG CGT AGG TCG CGT AAT TTG GGT GGA TCC TGG GAT ATA CTC TTC CCA
     GCC GCA TCC AGC GCA TTA AAC CCA CCT AGG ACC CTA TAT GAG AAG GGT
     Arg Arg Arg Ser Arg Asn Leu Gly Gly Ser Trp Asp Ile Leu Phe Pro

       390           400           410           420
        *             *             *             *
     GCC GAT CCG CCC TTT TCT CCC CAG AGT TGT CGA CCC CAG TGA
     CGG CTA GGC GGG AAA AGA GGG GTC TCA ACA GCT GGG GTC ACT
     Ala Asp Pro Pro Phe Ser Pro Gln Ser Cys Arg Pro Gln ***
```

Fig.13.

```
           10              20              30              40
            *               *               *               *
ATG AGT ACT AAC CCT AAA CCC CAA AGA AAA ACC AAA CGT AAC ACC AAC
TAC TCA TAG TTC GGA TTT GGG GTT TCT TTT TGG TTT GCA TTG TGG TTG
Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn

  50              60              70              80              90
   *               *               *               *               *
CGC CGT CCA CAG GAC GTC AAG TTC CCG GGC GGT GGT CAG ATC GTT GGT
GCG GCA GGT GTC CTG CAG TTC AAG GGC CCG CCA CCA GTC TAG CAA CCA
Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly

      100             110             120             130             14C
        *               *               *               *               *
GGA GTT TAC CTG TTG CCG CGC AGG GGC CCC AGG TTG GGT GTG CGC GCG
CCT CAA ATG GAC AAC GGC GCG TCC CCG GGG TCC AAC CCA CAC GCG CGC
Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala

          150             160             170             180             190
            *               *               *               *               *
CTC AGG AAG ACT TCC GAG CGG TCG CAA CCT CGT GGA AGG CGA CAA CCT
GAG TCC TTC TGA AGG CTC GCC AGC GTT GGA GCA CCT TCC GCT GTT GGA
Leu Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro

              200             210             220             230             240
                *               *               *               *               *
ATC CCC AAG GCT CGC CGA CCC GAG GGC AGG GCC TGG GCT CAG CCC GGG
TAG GGG TTC CGA GCG GCT GGG CTC CCG TCC CGG ACC CGA GTC GGG CCC
Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Ala Trp Ala Gln Pro Gly

                  250             260             270             280
                    *               *               *               *
TAC CCT TGG CCC CTC TAT GGC AAT GAG GGC ATG GGG TGG GCA GGA TGG
ATG GGA ACC GGG GAG ATA CCG TTA CTC CCG TAC CCC ACC CGT CCT ACC
Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Met Gly Trp Ala Gly Trp

290             300             310             320             330
  *               *               *               *               *
CTC CTG TCA CCC CGT GGT TCT CGG CCT AGT TGG GGC CCC TCA GAC CCC
GAG GAC AGT GGG GCA CCA AGA GCC GGA TCA ACC CCG GGG AGT CTG GGG
Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Ser Asp Pro

    340             350             360             370             380
      *               *               *               *               *
CGG CGT AGG TCG CGT AAT TTG GGT AAG GTC ATC GAT ACC CTT ACA TGC
GCC GCA TCC AGC GCA TTA AAC CCA TTC CAG TAG CTA TGG GAA TGT ACG
Arg Arg Arg Ser Arg Asn Leu Gly Lys Val Ile Asp Thr Leu Thr Cys

        390             400             410             420             430
          *               *               *               *               *
GGC TTC GCC GAC CTC ATG GGG TAC ATT CCG CTC GTC GGA TCC TGG GAT
CCG AAG CGG CTG GAG TAC CCC ATG TAA GGC GAG CAG CCT AGG ACC CTA
Gly Phe Ala Asp Leu Met Gly Tyr Ile Pro Leu Val Gly Ser Trp Asp
```

```
       440           450           460           470           480
        *             *             *             *             *
ATA CTC TTC CCA GCC GAT CCG CCC TTT TCT CCC CAG AGT TGT CGA CCC
TAT GAG AAG GGT CGG CTA GGC GGG AAA AGA GGG GTC TCA ACA GCT GGG
Ile Leu Phe Pro Ala Asp Pro Pro Phe Ser Pro Gln Ser Cys Arg Pro

CAG TGA
GTC ACT
Gln ***
```

Fig.14.

```
                 10              20              30              40
                 *               *               *               *
     ATG AGT ACT AAC CCT AAA CCC CAA AGA AAA ACC AAA CGT AAC ACC AAC
     TAC TCA TAG TTC GGA TTT GGG GTT TCT TTT TGG TTT GCA TTG TGG TTG
     Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn

     50              60              70              80              90
     *               *               *               *               *
     CGC CGT CCA CAG GAC GTC AAG TTC CCG GGC GGT GGT CAG ATC GTT GGT
     GCG GCA GGT GTC CTG CAG TTC AAG GGC CCG CCA CCA GTC TAG CAA CCA
     Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly

        100             110             120             130             140
        *               *               *               *               *
     GGA GTT TAC CTG TTG CCG CGC AGG GGC CCC AGG TTG GGT GTG CGC GCG
     CCT CAA ATG GAC AAC GGC GCG TCC CCG GGG TCC AAC CCA CAC GCG CGC
     Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala

        150             160             170             180             190
        *               *               *               *               *
     CTC AGG AAG ACT TCC GAG CGG TCG CAA CCT CGT GGA AGG CGA CAA CCT
     GAG TCC TTC TGA AGG CTC GCC AGC GTT GGA GCA CCT TCC GCT GTT GGA
     Leu Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro

           200             210             220             230             240
           *               *               *               *               *
     ATC CCC AAG GCT CGC CGA CCC GAG GGC AGG GCC TGG GCT CAG CCC GGG
     TAG GGG TTC CGA GCG GCT GGG CTC CCG TCC CGG ACC CGA GTC GGG CCC
     Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Ala Trp Ala Gln Pro Gly

              250             260             270             280
              *               *               *               *
     TAC CCT TGG CCC CTC TAT GGC AAT GAG GGC ATG GGG TGG GCA GGA TGG
     ATG GGA ACC GGG GAG ATA CCG TTA CTC CCG TAC CCC ACC CGT CCT ACC
     Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Met Gly Trp Ala Gly Trp

     290             300             310             320             330
     *               *               *               *               *
     CTC CTG TCA CCC CGT GGT TCT CGG CCT AGT TGG GGC CCC TCA GAC CCC
     GAG GAC AGT GGG GCA CCA AGA GCC GGA TCA ACC CCG GGG AGT CTG GGG
     Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Ser Asp Pro

        340             350             360             370             380
        *               *               *               *               *
     CGG CGT AGG TCG CGT AAT TTG GGT AAG GTC ATC GAT ACC CTT ACA TGC
     GCC GCA TCC AGC GCA TTA AAC CCA TTC CAG TAG CTA TGG GAA TGT ACG
     Arg Arg Arg Ser Arg Asn Leu Gly Lys Val Ile Asp Thr Leu Thr Cys

        390             400             410             420             430
        *               *               *               *               *
     GGC TTC GCC GAC CTC ATG GGG TAC ATT CCG CTC GTC GGC GCC CCT CTA
     CCG AAG CGG CTG GAG TAC CCC ATG TAA GGC GAG CAG CCG CGG GGA GAT
     Gly Phe Ala Asp Leu Met Gly Tyr Ile Pro Leu Val Gly Ala Pro Leu
```

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```
        440           450           460           470           480
         *             *             *             *             *
GGG GGC GCC GCC AGG GCC CTG GCG CAT GGC GTC CGG GTT CTG GAG GAC
CCC CCG CGG CGG TCC CGG GAC CGC GTA CCG CAG GCC CAA GAC CTC CTG
Gly Gly Ala Ala Arg Ala Leu Ala His Gly Val Arg Val Leu Glu Asp

        490           500           510           520
         *             *             *             *
GGC GGA TCC TGG GAT ATA CTC TTC CCA GCC GAT CCG CCC TTT TCT CCC
CCG CCT AGG ACC CTA TAT GAG AAG GGT CGG CTA GGC GGG AAA AGA GGG
Gly Gly Ser Trp Asp Ile Leu Phe Pro Ala Asp Pro Pro Phe Ser Pro

530           540
 *             *
CAG AGT TGT CGA CCC CAG TGA
GTC TCA ACA GCT GGG GTC ACT
Gln Ser Cys Arg Pro Gln ***
```

Figure 15: *In vitro* Translation of RNA from pδC27core 120a
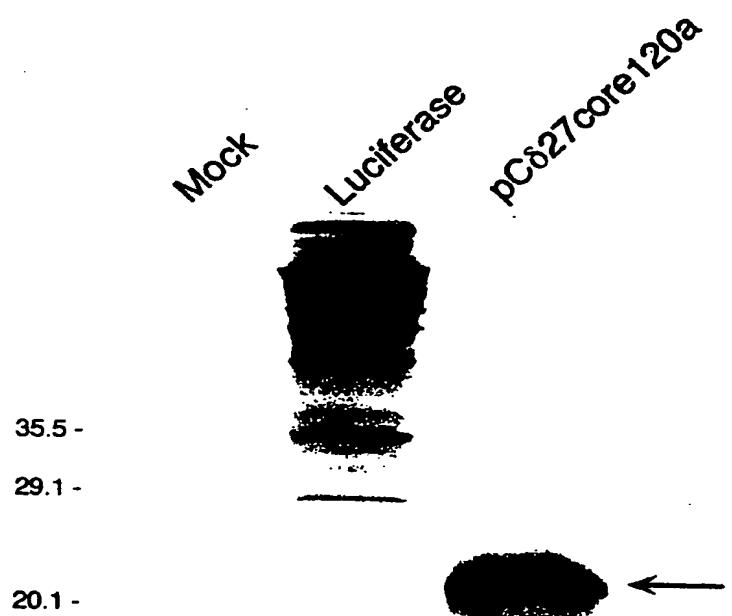
Fig 15,

Figure 16: Western Blot of *in vitro* translated RNA from pCδ27core120a

Fig.16.

Fig. 17

CMV
Promoter
        HCV Core aa 1-140   L-HDAg aa 1-214

pC c140 FLδ.

CMV
Promoter
        Full Length HCV Core   L-HDAg aa 1-214

pC FL core+δ.

HCV core aa1-40   HCV core aa1-40

L-HDAg   N ——— Apa1 ——— Sma1 ——— C

Coiled Coil Domain   Core NLS   ARM 1   ARM 2   Proline/Glycine-Rich Domain

Whole NLS   RNA Binding Domain   Prenylation Site
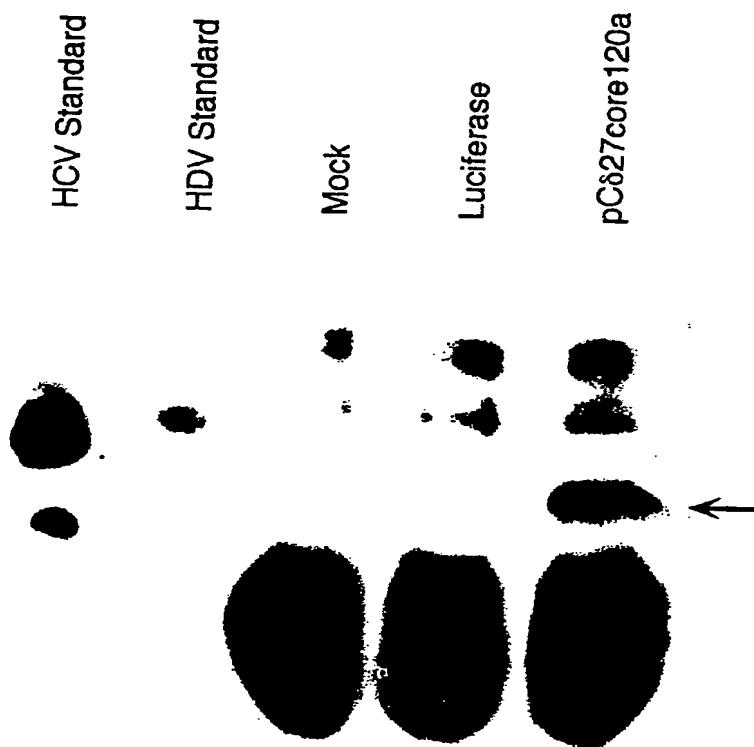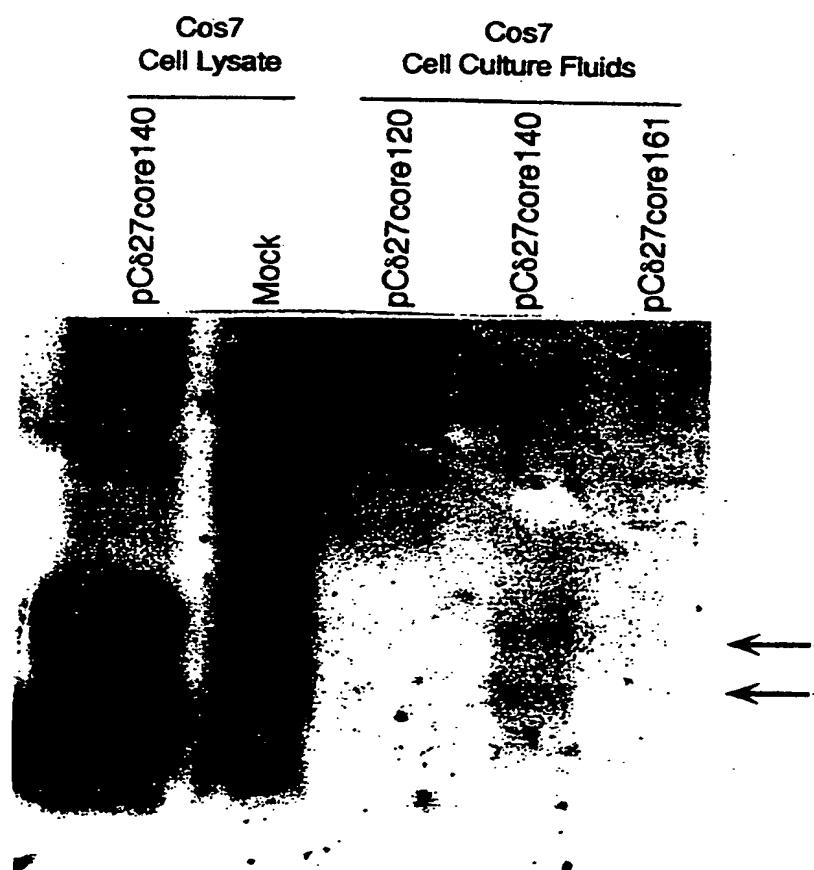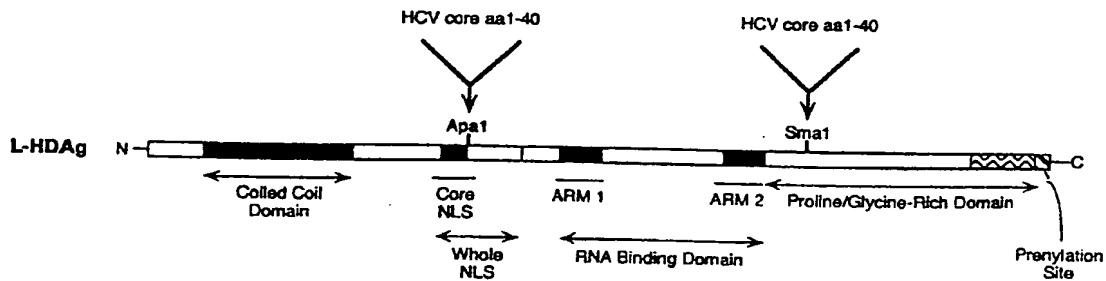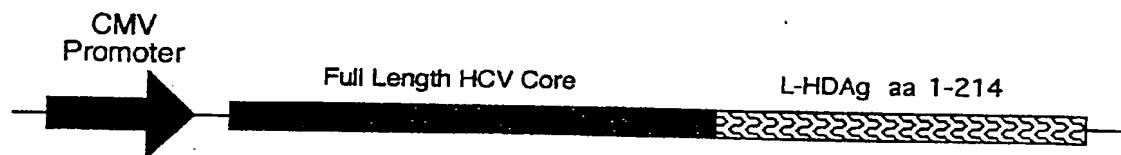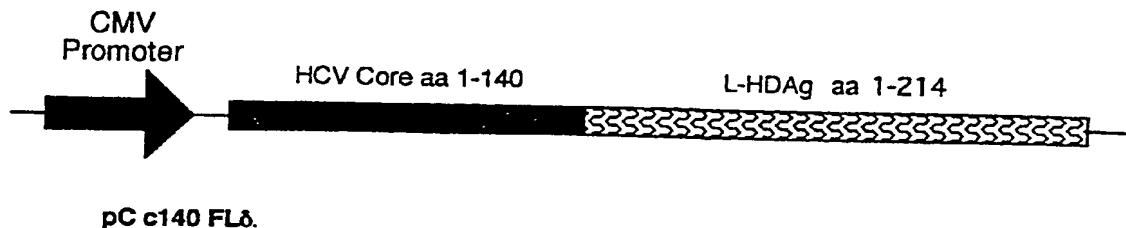
pSV27-C40 Apa I / Sma I

Fig.18.

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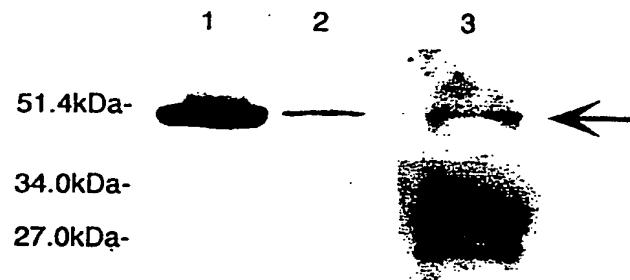
Fig.19

51.4kDa-

34.0kDa-

27.0kDa-

Fig 20.

Fig. 21

Fig 22.

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]:   A61K 39/29, C12N 7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int Cl[6]: A61K 39/29, C12N 7/01

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
(see attached)

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	WO 91/04319 A1(INNOVIR LABORATORIES) 4 April 1990; SEE EXAMPLES 2 and 5 in particular.	1-20
Y	Derwent abstract accession no. 95-390238/50 and JP 07-267878 (KOGAWA A) 17 October 1995. See abstracts.	1-20
X, Y	Lee, C. et al. (1995) Large Hepatitis Antigen in Packaging and Replication Inhibition: Role of the Carboxyl - Terminal 19 Amino Acids and Amino-Terminal Sequences, *Journal of Virology*, vol 69 no. 9, 5332-6. See page 5335 final paragraph in particular.	1-20

[X] Further documents are listed in the continuation of Box C		[X] See patent family annex

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such
"O"	document referring to an oral disclosure, use, exhibition or other means		combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 February 1998	6 MAR 1998

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	David Hennessy Telephone No.: (02) 6283 2255

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INTERNATIONAL SEARCH REPORT

C (Continuation)	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	Chen, P. et al. (1993) Hepatitis Surface Antigen and Large-Form Hepatitis Delta Antigen in HDV Assembly: A further Study, *Progress in Clinical and Biological Research*, vol. 382, 29-34. See page 33 in particular.	1-20
X, Y	Chen, P. et al. (1992) functional study of Hepatitis Delta Virus Large Antigen in Packaging and Replication Inhibition: Role of the Amino-Terminal Leucine Zipper, *Journal of Virology*, vol. 66 no.5, 2853-9. See discussion in particular.	1-20
Y	Sheu, S.Y. et al. (1996) No Intermolecular Interaction between the Large Hepatitis Delta Antigen is Required for the Secretion with Hepatitis B surface Antigen: A model of empty HDV Particle, *Virology*, vol. 218, 275-8. See whole article.	1-20
Y	Lee, C. et al. (1994) Isoprenylation of Large Hepatitis Delta Antigen is Necessary but not sufficient for Hepatitis Delta Virus Assembly, *Virology*, vol. 199, 169-75. See whole article	1-20
Y	Wang, H. et al. (1994) Packaging of Hepatitis Delta Virus RNA via the RNA-binding Domain of Hepatitis Delta Antigens: Different roles for the small and large Delta Antigens, *Journal of Virology*, vol. 68 no. 10, 6363-71. See whole Article.	1-20

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INTERNATIONAL SEARCH REPORT

Box (B49)

DATABASE: DERWENT WPAT, JPAT KEYWORDS:

SS 1: C12N/IC AND A61K/IC
SS 2: HEPATITIS (W)D OR HEPATITIS (W) DELTA OR HDV
SS 3: HEPATITIS (W)B OR HEPATITIS (W) BETA OR HBV
SS 4: HBSAG OR HEPATITIS (W) B (W) SURFACE (W) ANTIGEN
SS 5: CAPSID# OR VIRON# OR VIRION #OR PARTICLE# OR VIR:(W) ASSEMBL:

DATABASE: MEDLINE AND CHEMICAL ABSTRACTS (CA)
KEYWORDS:
DELTA AGENT/CT
HEPATITIS DELTA
VIRION OR PACKAG? OR ASSEMBL? OR PARTICLE# OR CAPSID
HEPATITIS B SURFACE ANTIGENS

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INTERNATIONAL SEARCH REPORT
Information on patent family members

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9104319	AU	65059/90	AU	65115/90	AU	78931/94
		AU	658129	AU	674104	CA	2066647
		CA	2066684	EP	494228	EP	494244
		JP	5502999	JP	5503838	KR	9612066
		KR	9705045	US	5225337	WO	9104319
		WO	9104324	US	5225347		

END OF ANNEX

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